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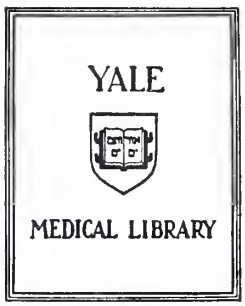
THE TREATMENT OF PERIPHERAL NERVE INJURIES WITH CRYOPRESERVED AUTOGRAFTS A FUNCTIONAL, HISTOLOGICAL, AND BIOCHEMICAL EVALUATION OF THE ROLE OF CRYOPRESERVATION IN THE RAT MODEL

PATRICK ALAN HOWE

1987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THE TREATMENT OF PERIPHERAL NERVE INJURIES WITH CYROPRESERVED AUTOGRAFTS. A FUNCTIONAL, HISTOLOGICAL, AND BIOCHEMICAL EVALUATION OF THE ROLE OF CRYOPRESERVATION IN THE RAT MODEL.

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

PATRICK ALAN RUWE

1987

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ABSTRACT

THE TREATMENT OF PERIPHERAL NERVE INJURIES WITH CYROPRESERVED AUTOGRAFTS. A FUNCTIONAL, HISTOLOGICAL, AND BIOCHEMICAL EVALUATION OF THE ROLE OF CRYOPRESERVATION IN THE RAT MODEL.

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Adult male Sprague-Dawley rats were used as a model to study the treatment of peripheral nerve injuries with cryopreserved autografts. Three experimental groups were used. The first control grafted group (n=11) recieved a 2.0 cm fresh autograft. The second cryopreserved group (n=12) recieved a 2.0 cm cryopreserved graft. The third gap nongrafted group (n=13) was left with a 2.0 cm deficit in one sciatic nerve. Histological, functional, and biochemical evaluations of the role of cryopreservation were undertaken.

Results showed a mean fiber density of myelinated axons in the fresh autograft group of 5,025 (SD,457) and in the cryopreserved autograft group of 5,126 (SD,297). The normal mean value for this segment of nerve was 3,885 (SD,744). In functional tests the fresh grafted animals had a mean flexion contracture of 18^{o} compared to 14^{o} in the cryopreserved animals. The gap control animals had a mean flexion contracture of 49^{o}. The mean weight for a tibialis anterior (TA) muscle innervated by a fresh nerve graft was .5877 grams (SD,.0746) and innervated by a cryopreserved nerve graft .5801 grams (SD,.0935). The mean weight of a TA muscle in the gap control group was .2089 grams (SD,.0844). The mean hydroxyproline (HP) percentage in TA muscle innervated by a fresh graft expressed as op/control leg was 1.527 and innervated by a cryopreserved graft 1.660. The mean HP ratio in gap control animals was 4.315.

Our results have shown that fresh and cryopreserved autografts are not statistically different in their ability to potentiate regeneration of axons and return of function in the rat. They are however, superior in this ability to gap control animals. These findings have immediate clinical applicability in cases of segmental nerve loss in a wound not immediately ready for grafting or in a patient not yet stabilized for major sugery.

DEDICATION

To Mom and Dad

and to Stacy for her

understanding and support.

ACKNOWLEDGEMENTS

The Author is greatly indebted to Mrs Gertrude Chaplin, Medical Histologist, for her invaluable work in the preparation of histological sections for this study. In addition, I am much obliged to Ms. Sarah Whitaker, Medical Photographer, for her help in preparing illustrations, Dr. Karen Edwards for her help in the statistical analysis of the data and Alice Gorton for her help in manuscript preparation.

Special thanks to Dr. Thomas E. Trumble my teacher and friend who gave unselfishly of himself in introducing me to the world of investigative Orthopaedics.

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I. Introduction

Physicians have long dreamed of one day having the ability to replace worn, diseased or damaged human body parts with donor tissue. Medical researchers have made great advances in the field of organ transplantation with respect to kidney, heart, liver, bone, and corneal transplantation. Unfortunately, the successful transplantation of nervous tissue and specifically peripheral nerves has lagged far behind the rest.

The repair of large defects in peripheral nerves after traumatic injury is a frequent and frustrating problem in surgery of the extremities. Often, as the result of an accident, a patient is left with an anatomically intact distal extremity without adequate motor or sensory innervation to allow satisfactory function.

Currently, peripheral nerve injuries are treated using autografts such as the sural nerve which are harvested from the patient at the time of reconstructive nerve surgery [16,19]. Several problems make this approach to the treatment of peripheral nerve injuries less than optimal. Unlike other autografts such as vein and bone grafts, the removal of nerve to obtain tissue for autogenous nerve grafting leaves a permanent neurologic deficit. Furthermore, the amount of donor tissue is inherently limited. If it were possible to use cadaveric allografts, the aforementioned problems would be averted.

Two major difficulties must be overcome for the allogeneic nerve tissue approach to be effective. Firstly, immunosuppression may be required. Secondly, optimal methods for storing or banking nerve tissue must be determined. A great deal of work has and continues to be directed toward the question of immunosuppression. Zalewski et al. [119,120] have demonstrated that allogeneic nerve tissue is antigenic and that the immune response in the recipient animal can be altered by cyclosporin-A. In addition, several investigators have tested various methods for decreasing the antigenicity of the nerve tissue itself [36,59,60,61,64,81,97,100,118]. It is the purpose of this thesis to study the latter question concerning optimal methodology for storing or banking nerve tissue without which allogeneic nerve grafting would be much less practical. Specifically, this paper will address the treatment of peripheral nerve injuries with cryopreserved autografts evaluating the role of cryopreservation in the rat model. A comparison will be drawn between fresh and cryopreserved autografts and interpreted with respect to functional tests, sensory tests, axonal counts, and hydroxyproline assays. The use of autografts has been chosen in a specific attempt to minimize the role of the immune response in this model [125].

The clinical need for a system for banking peripheral nerve is obvious. As previously stated, allogeneic nerve grafts harvested from cadaveric donors would require

cyropreservation to be practical. But cryopreservation and peripheral nerve banking also has an immediate and proven clinical application with respect to autogenous tissue. The ability to bank autogenous nerve tissue would be invaluable in the care of the major trauma patient with multiple limb injuries. Viable nerve tissue could be harvested in an unsalvagable or amputated limb and stored for delayed repair of segmental nerve loss in a wound not immediately ready for grafting or in a patient not yet stabilized for major surgery. In addition, proven cryopreservation technique would afford the surgeon the option to perform reconstruction in an elective rather than emergent setting and thus avoid many of the problems associated with emergency surgery.

It is the authors' hope that this work will take a small step toward establishing the role of cryopreservation in peripheral nerve grafting and in so doing potentiate a larger step toward the dream of using allogeneic tissue to repair peripheral nerve defects in man.

II. Review of the Literature

A. Historical Perspective of Nerve Grafting

Physicians have been experimenting with nerve grafting, allografting, autografting, and xenografting for over one hundred years. Peripheral nerve allografting started experimentally in 1870 with the now classic work of Philipeaux and Vulpian reported in the French literature [78]. They reported results showing that a graft could conduct nerve fibers across a defect. Other early authors were able to duplicate their results showing that grafted pieces of nerve could be used to conduct nerve fibers across peripheral nerve lesions [7,17,41]. The first clinically reported allograft in man was reported by Albert [3] in 1878 in the German literature. He described a procedure by which he transplanted an allograft nerve segment from an amputated limb to bridge a 3.0 cm deficit of the median nerve in the recipient's forearm created as the result of the resection of a sarcoma. This patient, however, never recovered function in the limb. The first reported clinical allografting in the English literature appeared in 1889 in the form of a paper read before the Clinical Society of London by Mayo-Robson [66]. He described the case of a fourteen year old girl who was left with a two inch deficit in her median nerve as the result of tumor surgery with resultant loss of function in her affected hand. Repair of the median nerve was effected by

grafting a 2 1/2 inch segment of the posterior tibial nerve harvested from the freshly amputated leg of another patient. One might question Mayo-Robson's results as he goes further to describe return of sensation within thirty-six hours and gradual return of function in the paralyzed muscles [66]. Perhaps encouraged by his earlier reported successes, Mayo-Robson in 1890, used the sciatic nerve of a rabbit to bridge a gap in the ulnar nerve of a gardener who had lacerated his forearm with a scythe. Faced with a 2 1/2 inch deficit in the median nerve of the same patient and having previously exhausted his supply of rabbit sciatic nerve he dissected free and interposed a segment of the rabbit's spinal cord into the gap. Mayo-Robson further described complete functional recovery in this patient after several years.

Unfortunately, Mayo-Robson's successes have not been verified in the laboratory. Huber [41], in 1895, reported early success in grafting the sciatic nerves of cats into dogs but twenty years later as the result of more extensive and controlled work concluded that xenografts were less successful than autografts or allografts. Eden [31], in 1919, and Cajal [17], in 1928, condemned the use of xenographs as a result of their own separate investigations and with respect to careful review of the current literature of the day.

Concurrent with the condemnation of heterografts many authors were finding experimentally reproducible success as

judged by recovery of sensation and motor function with autografts in both animals and man [10,17,30,31,35,43].

Foerster [35] reporting in the German literature in 1929 described his series of twenty-one autografts. Of these cases, five patients experienced complete recovery of both sensory and motor function. Another twelve patients experienced partial return while only two cases were judged as failures and two cases were lost to follow-up. Nerve autografting, however, did not gain universal popularity and approval until the work of Ballance and Duel [7] was published in 1932 in the Archives of Otolaryngology. They reported on the success of the operative treatment of facial palsy by the use of autografts to repair the facial nerve. Concomitantly with Ballance and Duel's work, Bunnel and Boyes [15] described the successful use of autografts to repair digital nerve defects in their series of thirty-two patients.

Through the work of these and other investigators the efficacy of autografting was established almost fifty years ago. Unfortunately, as previously mentioned the use of autografts is inherently limited by the supply of tissue and by the creation of permanent neurologic deficits at the donor site. These factors are magnified in the case of a lesion involving a long segment of nerve or a large diameter axon and limit the clinical application of autografts in humans.

The use of allografts in the repair of peripheral nerve

lesions would appear as the ideal solution. A large defect in the median nerve could be repaired with an anatomically similar segment from the median nerve of a cadaveric donor without the recipient incurring further neurologic loss.

Some early authors reported initial success with allografts in the animal laboratory [31,43,49]. However, their transplantation technique, experimental methodology, and interpretation of results were inconsistent and not fully described. In 1918, Verga [108] was among the first to demonstrate through histology that an allograft could at first unite with the host tissue but later degenerate. Eden [31] confirmed these results a year later showing again histologically that axons from the proximal host stump could grow a short distance into the allograft but more distally would be replaced by fibrous scar tissue. In his experimental model, Eden attempted a 5.0 cm allograft in the sciatic nerve of a dog. Histological examination revealed sparse axonal regeneration in the proximal graft while the remainder of the graft was largely replaced by fibrous tissue. As the result of independent research, Stookey [102] and Huber [43] concluded that this dense fibrous tissue which formed in response to the allograft acted as a mechanical barrier to the regeneration of the nerve axon down the allograft.

In an attempt to standardize the assessment of the efficacy of the various types of grafting procedures in light of the often contradictory findings in the literature

of the day and to address the issue of fibrous replacement of grafted nerve segments, Sanders and Young [90], in 1942, compared the results of nerve grafting with fresh and predegenerated autografts, fresh and pretreated allografts, and fresh and pretreated xenografts. They, along with Guttman and Sanders [39], took the various types of nerve grafts and inserted them into the peroneal nerve of the rabbit. Dog and rat nerve segments were tested in xenograft experiments. After fifteen to twenty-five days and sometimes longer, examination was made of the histological changes in the graft and the distance of outgrowth of host axons. The authors concluded that the cells of the allograft were destroyed as a result of a host immune reaction, the severity and time of commencement of which was determined by the genetic relationship of the host and the donor and the amount of tissue transplanted. These immune reactions were even more pronounced in the xenografted animals. They further demonstrated that the Schwann cells of an autograft survive and multiply setting up a channel for sprouting axons thus effecting a good basis for recovery.

Around the time of Saunders and Young's classic work two clinical allograft series appeared in the literature. Seddon and Holmes [93], in 1944, unsuccessfully attempted four human allografts which upon subsequent exploration revealed total replacement by fibrous tissue. In 1945, Spurling et al. [99] reported the results of their series

of eight cases of human allografts all of which on re-exploration revealed replacement by fibrous tissue. This basic problem of inflammatory response and fibrous replacement of allograft tissue first described by Saunders and Young and now known to be the result of the immunological rejection of foreign proteins by the host, renders any type of peripheral nerve allograft procedure impractical unless an alteration in immune response can be found. It is in this direction that the current scope of peripheral nerve allograft research is directed. The results of Sanders and Young's attempts to alter the immune response in their classic article by predegeneration, formol fixation or alcohol saturation were equivocal. But clearly, they set the tack for future investigations.

B. Contemporary Progress in Peripheral Nerve Allografting

Since the early work of Sanders and Young elucidating the immune reaction to peripheral nerve allografts, research has been directed first toward decreasing the antigenicity of the nerve graft itself without altering its physiological properties and later, with the advent of immunosuppressive pharmacological agents and a greater understanding of the immune system, toward alteration of the host response.

Early attempts at altering the immunogenecity of allograft segments met with limited success. Cajal [17],

in 1928, was among the first to propose predegeneration of nerve graft segments on purely theoretical grounds. In 1933, Duel [30] published the results of his work in the laboratory with predegenerated autografts. Huber [43] attempted, in 1920, to store nerve tissue in vaseline with limited success. Cajal [17] concluded that grafts treated by boiling water, chloroform, formalin, and chloral hydrate were not innervated and that dead grafts had limited clinical application.

In the past twenty years, a select number of investigators have contributed markedly to the field of peripheral nerve allograft research. Stimulated by previous clinical success with cathode-irradiated allogeneic bone grafts and from information gained as the result of previous studies of successful cathode-irradiated allogeneic tendon grafts in chickens, Leonard Marmor [64], in 1964, pioneered the use of electron radiation to reduce the foreign body reaction well described in the literature in response to allogeneic nerve grafts. Campbell and Bassett [126] had, in 1959, described the use of irradiation in peripheral nerve grafts, but had done so in combination with the use of a millipore tube, a cellulose-acetate filter which acts as a cell barricade while allowing the defusion of tissue fluids to the nerve grafts. They, however, attributed their successful reduction of the immune response to the millipore filter. Reporting at the 1962 meeting of the American Academy of

Orthopaedic Surgeons, Marmor described his implantation of irradiated allogeneic nerve segments in one thigh and non-irradiated allogeneic nerve segments in the opposite thigh of two isogeneic strains of rats which differed with respect to both minor and major transplantation antigens. Two weeks after implantation, the irradiated nerve graft was intact with no evidence of local immune reaction. By contrast the non-irradiated graft had been invaded by inflammatory cells resulting in marked fibrosis and destruction of the graft. Similar results were seen at six weeks. Marmor concluded that irradiation drastically reduced the foreign body rejection phenomena and set up an experiment to determine whether long nerve defects could be bridged successfully with irradiated allografts employed in a physiological manner.

In his series of three mongrel dogs, Marmor repaired a 3.0 cm deficit in the peroneal nerve with a 3.5 cm irradiated allograft. Three weeks after the operation, electromyograms revealed complete denervation of the dogs' dorsiflexor muscles of the foot and physical exam revealed a foot drop and a limited gait in each of the animals. At six months, Marmor reported normal EMGS, resolution of foot drop, and normal gait with no evidence of weakness in all three dogs. Histological exam revealed regenerated axons in and distal to the graft with minimal fibrous tissue proliferation [64]. Marmor's experimental findings could have been strengthened by the use of more animals and by

the use of a group which received non-irradiated grafts and a group which received no graft at all as a check for spontaneous regeneraton. In addition, the choice of mongrel dogs caused the animals' antigeneic disparity to be unknown.

In his book entitled Peripheral Nerve Regeneration Using Nerve Grafts published in 1967 [125], Marmor reported his clinical results using irradiated allografts to repair peripheral nerve defects in twenty-five humans from 1961 to 1966. The grafts which Marmor inserted ranged in length from 4.5 to 22.0 cm. Some patients recovered sensation and two experienced some subjective return in motor function, but the great majority of grafts were unsuccessful. In his book Marmor concludes that successful allografts would require further protection against destruction in addition to irradiation and proposed the use of suppression of the host response by systemic drugs.

In later work, Marmor et al. [65] describe an experiment in rats designed to study the effect of nontoxic doses of the immunosuppressive drug Imuran on homotransplantation of peripheral nerves, both alone and in combination with graft irradiation. Repeating nonphysiological techniques used in previous experiments [64], the authors concluded as a result of histological findings that the immune rejection response to nerve allografts can be prevented in animals by the administration of Imuran in nontoxic doses [65]. As in

earlier experiments, the authors chose to use a very limited number of animals (in this case a total of ten with two animals sacrificed at five one-week intervals). In addition, unlike in their previous study, the authors used Sprague-Dawley rats, an outbred strain with an unknown histocompatibility complex, for their model and again failed to report data from a control group which would receive untreated nerves and no immunosuppression. Nevertheless, Marmor's work represents the first attempt to combine a technique to decrease the antigenecity of the allograft nerve segment with systemic immunosuppression of the host.

Ducker and Hayes [29], in 1970, reported their results from attempts to decrease the allograft antigenecity. They attempted to duplicate Marmor's work with irradiation while also testing the effect of Wallerian degeneration on the immune response. No attempt was made to alter the host immune reaction with systemic immunosuppressive drugs. They avoided the problems Marmor had with experimental design by replacing a 2.0 cm nerve segment in dogs with 1) a control measured gap with nerve ends sutured to fascia, 2) an autograft nerve segment, 3) a predegenerated allograft (three weeks), and 4) an irradiated allograft. Each experiment was done using grafts of two different lengths (2.0 cm, 4.0 cm). Measurements of threshold stimuli and conduction times across the graft were used to judge results at six months. No attempt at functional

evaluation was undertaken. The results showed unsuccessful reinnervation in both the gap and predegenerated grafts. The 2.0 cm autografts and irradiated allografts were highly successful. The 4.0 cm irradiated graft was marginally successful. By histological analysis, the authors showed that in the predegenerated grafts, an inflammatory response created thick scar tissue and blocked axons from growing into the graft. In earlier studies involving chimpanzees receiving allografts of varying lengths (2.0 - 7.0 cm) which had been both predegenerated and irradiated, they found 80 percent success in grafts under 4.5 cm in length while only 33 percent success in longer grafts [29]. It should be noted again that no functional studies were performed. From their data the authors concluded that the best nerve graft was an autograft of less than 4.0 cm in length (90 percent effective). The maximum length practical for allografts treated by irradiation and degeneration in higher primates was also 4.0 cm (80 percent effective) [29].

Buch [13] in an attempt to duplicate the earlier results of Marmor [64,65] studied the effect of pretreatment with irradiation in guinea pigs and dogs. He concluded that prior radiation exposure of nerve allografts minimizes but does not totally obviate the inflammatory response or subsequent fibrosis.

In order to explore the exact mechanism for the now established beneficial effect of exposing frozen nerve

segments to extremely high levels of radiation [13,29,64,65] Verhoog and van Bekkum [109] devised an experimental model consisting of heterotopic transplantation of peripheral nerve segments under the kidney capsule of rats. They graded their results by counting the infiltrated round cells in stained histological sections of the graft. As a result of low dose radiation (1500, 15,000 rads) they found little inhibition of the lymphocytic response, but complete absence of this response in grafts receiving high dose radiation (150,000 rads). Similar results were seen with exposure to increasing concentrations of the cytotoxic agent Cialit. They further showed that freezing to -192^{o} C without the cyroprotectant agent DMSO also inhibited the immune response while cryoprotection restores the tendency for lymphocytic infiltration. They concluded that transplantation antigens responsible for the allograft response are expressed only by living cells, and therefore, cell death was the responsible mechanism for inhibition of immune response secondary to irradiation [109].

J.D. Pollard [80,83] and his associates working in Australia reported in 1973 results from their work with respect to ultrastructural comparisons and classification of general host response to peripheral nerve allografts and autografts. They further performed a physiological study involving their attempt to limit the immune response in

peripheral nerve allografts by means of Imuran therapy. With regard to the host response, Pollard characterized the allograft response on the cellular level as an invasion of graft tissue by lymphocytes, plasma cells, and activated macrophages which disrupt the normal nerve architecture causing rupture and subsequent compression of neurolemmal tubes, thus, effectively blocking the conduit for axonal sprouting [80]. He describes this progressive obliteration as beginning three weeks after grafting and leading to large areas of collapsed neurolemmal tubes embedded in collagen by four months. He further classifies three major distinguishing features between autografts and allografts. Firstly, the lymphocyte and plamsa cell infiltration is completely absent in the autograft. Secondly, the dominant cell within the neurolemmal tube in the autograft was the Schwann cell whereas in the allograft it was the macrophage. In fact, there was no evidence of Schwann cell survival at all in the allograft. Thirdly, whereas the neurolemmal tube was shown to survive for long periods of time and to conduct a column of Schwann cells and regenerating axons to the periphery in autografts in the allograft the majority of neurolemmal tubes were as previously mentioned collapsed and compressed by surrounding collagen. Pollard proposes that successful results in peripheral nerve allografts are dependent on immune suppression of this destruction of neurolemmal tubes [80].

To test his theory, Pollard set up an experimental allograft model utilizing forty-two Wistar rats. Unfortunately, he failed to identify the strain of rat which served as the nerve donor so we have no information regarding histocompatibility differences. One half of the rats received Imuran in nontoxic doses during the regeneration period. Results were judged to be significantly better in the Imuran treated group at six months with respect to motor conduction velocity and density of myelinated fibers within and distal to the graft [83]. The authors concluded that treatment with Imuran lessens the intensity of the immune response responsible for rupture and collapse of neurolemmal tubes [80] allowing the regeneration through nerve allografts to occur more readily [83] in the treated group.

In an attempt to identify the constituents of peripheral nerve allografts which evoke an immune response Pollard and McLeod [81] investigated the fate of donor Schwann cells by inserting nerve grafts into trembler mice which have a hereditary demyelinating neuropathy allowing easy distinction between host and donor Schwann cells. By using predegenerated grafts devoid of myelin, the authors proposed by electrophysiological and histological criteria that when significant histocompatibility differences existed, Schwann cells within nerve allografts were rejected irrespective of the presence or absence of myelin within the nerve graft. The authors concluded that Schwann

cells, not myelin, constitute the principal antigen within nerve autografts and that their rejection is responsible for neurolemmal tube collapse, thus, limiting the success of nerve allografts. It is difficult to draw conclusions by the above data and without description of statistical analysis. One cannot conclude by showing in a multidimensional model, in this case a peripheral nerve allograft, that grafts were rejected whether or not they contain myelin that donor Schwann cells are responsible for this immune response. Evidence could be gained by selectively killing allogeneic Schwann cells before grafting and showing resultant inhibition of the immune response. Clearly, more work is needed in this area.

Levinthal [53] reporting results from a comparative experiment orthotopically transplanted nerve fascicles between different strains of antigenetically identified rats [74]. He could not differentiate an allografted fascicle from its autografted control as judged with respect to cellular reaction, macrophage response, connective tissue proliferation, distal myelination or estimated axonal ratios. Levinthal et al. concluded that the major transplantation antigens responsible for the immune response are predominantly expressed in the epineural and surrounding connective tissues, while fascicular or perineural elements of the grafts are less antigenic [53].

In an attempt to show that histocompatibility plays a

major role in the results of peripheral nerve surgery, Bucko and Steinmuller [14] , reported in 1974 their results using 3.0 cm isografts, allografts, and xenografts in highly inbred rats. They found reinnervation of denervated rat tails in ten of eleven trials with isografts, two reinnervations in fifty-nine rats receiving allografts pretreated by irradiation, freezing, lyophilization, predegeneration, or a combination of the above, and total failure in all cases of fresh allografts or xenografts. By using statistically significant numbers of animals and strict experimental design, they concluded that although the possibility of unknown non-immunogenic factors existed, transplantation immunity played the major role in preventing regeneration in the allogeneic model [14].

With the role of histoincompatibility in the prevention of regeneration in peripheral nerve allografts gaining acceptance in the literature, Singh, Melchese, and Stefanko [94,95] reporting from the Netherlands were among the first to investigate the influence of tissue typing on nerve regeneration. From their study involving thirty-six dogs tissue typed with respect to the major histocompatibility complex system and broken down into three major groups (group 1 receiving a 4.0 cm graft; group 2, a 4.0 cm graft subjected to radiation; and group 3, a 7.0 cm graft), they concluded that compatible tissue typed nerve allografts behave more like autografts and are, therefore, more readily accepted by the host tissue. Unfortunately, their

experimental model was fraught with problems. Firstly, the authors failed to describe the exact histocompatibility differences between the dogs used for allografting as determined by tissue typing. Secondly, they subjected all nerve grafts to freezing to -70^{0} C for four to six weeks without a cryoprotectant agent prior to implantation. Verhog and von Bekkum [109] had shown earlier that freezing without a cryoprotectant agent inhibited the immune response by killing the cells which carry the transplantation antigens. Furthermore, Singh et al. had no control group to present data as to whether fresh nerve allografts were rejected. This would help to clarify the dogs' antigenic differences and to help elucidate the role of cryopreservation. Thirdly, the authors failed to describe the level of radiation used in their experiments. Marmor [64] had in earlier work shown that low dose radiation had a limited effect on the immune response while high dose radiation suppressed the response by killing the cells. Singh et al. had presumably already killed the cells by freezing them without a cryoprotectant agent [109]. In the interpretation of their results Singth et al. [94,95] used the maximum nerve conduction velocity and a subjective interpretation of histological findings as the criteria for judging their results. Even with such criteria their results were not statistically significant when they compared compatible and noncompatible 4.0 cm grafts without radiation treatment.

In a later project, Singh et al. [97] studied the histocompatibility requirements for successful frozen nerve allografts in forty-six dogs. In this project they avoided some of their earlier experimental design problems by describing exactly the number of sites on the major histocompatibility complex in which each dog differed. Further, they described the exact dose of radiation used in the pretreatment of the graft. Again, however, they cryopreserved all grafts without a cryoprotectant agent and failed to present data involving fresh nerve allografts as controls. Using their previously described criteria [94,95] to judge the results Singh et al. [97] concluded that minor histocompatibility differences did not lead to rejection of the frozen nerve allografts even in grafts of 7.0 cm length. In fact, Singh et al. reported that five out of six dogs receiving 7.0 cm allografts recovered completely normal conduction velocities. In previously reported more controlled studies, Ducker and Hayes [29] had shown that even in 4.0 cm autografts in dogs conduction times in the best results were decreased by 15 percent. Furthermore, Ducker and Hayes found vascular insufficiency to be a limiting factor in autografts over 4.0 cm. Clearly, Singh et al. findings would be dramatic if their results could be reproduced under more controlled conditions.

In a direct attempt to provide confirmation that host axons can regenerate through a long nerve allograft

provided that the allograft bears only minor transplantation antigens and is frozen and thawed before transplantation, Zalewski and Gulati [118] working at the National Institute of Health reported, in 1982, the results of their research using the rat model. In their model, host isogeneic Fisher rats received a 4.0 cm fresh or frozen allograft from isogeneic Lewis rats. Fisher and Lewis rats are known to differ only with respect to minor transplantation antigens [74]. Grafts were frozen in exactly the same manner as in Singh et al.'s [97] experiment without the use of a cryoprotectant agent. Histological examination showed that at two and nine months only the fresh isograft permitted host axon regeneration. Only a few axons regenerated into the most proximal portion of either the frozen isografts or the fresh allograft. Histological examination of separate specimens after two weeks revealed that freezing killed most of the Schwann cells [118] as had been reported earlier [29]. The authors concluded that axonal regeneration through a long frozen nerve graft fails in rats because freezing destroys Schwann cells [118]. Clearly Zalewski and Gulati's findings failed to confirm Singh et al.'s work in dogs.

In further work, Zalewski and Silvers [129] avoiding the unclear role of cryopreservation investigated whether a fresh nerve allograft containing only minor transplantation antigens would be superior to one containing minor and major incompatibilities in both normal and immunologically

tolerant rats [121]. The author's experimental model involved the grafting of the peroneal nerve in rats. Success of the nerve graft was evaluated by determining whether host motor nerve fibers grew through the nerve graft to reinnervate the tibialis anterior (TA) and extensor digitorum longus (EDL) muscles. Rats were checked clinically for foot drop as well as for evidence of muscular atrophy [121]. They found that nerve fibers were able to regenerate functionally through 2.0 cm and 4.0 cm isografts and through 2.0 cm allografts containing minor or minor and major histocompatibility differences. The 4.0 cm allografts failed irrespective of histocompatibility. The 2.0 and 4.0 cm allografts were, however, successful when transplanted into immunologically tolerant rats. Zalewski and Silvers classified histologically successful grafts as having sometimes greater or sometimes less axons than the proximal stump, but always having axons of thinner diameter [121]. The authors conclude that even minor antigens can evoke an immune response sufficient to prevent functional regeneration through a long length of nerve allograft. From the results of the immunologically tolerant group, they concluded that host immunosuppression offers the best hope of achieving regeneration through a long nerve allograft [121]. Again, Zalewski's work contradicts Singh et al.'s conclusions [97]. Unfortunately, there were problems with Zalewski and Silver's experimental design. To achieve a 4.0 cm graft, the authors combined two-2.0 cm

graft segments, thus, requiring regenerating axons to cross three suture lines rather than two. deMedinaceli [24,25,26], and others [94,95] have shown that the resultant blood clot and cellular debris at a suture line can be a critical factor in determining whether axons can regenerate across lines of union. The authors failed to report any histological findings with respect to the suture lines. In addition, with respect to allografts involving immunologically tolerant rats, results were reported only in animals who differed with respect to minor histocompatability antigens. Since the controversy as to the role of minor HLA differences in graft rejection was, in fact, the focus of their work, conclusions could have been stronger if additional data had been reported. Specifically data, with respect to allografts, between immunologically tolerant animals which differed with respect to major antigens was needed.

Clearly, controversy exists in the literature with respect to the role of minor transplantation differences in the rejection process of peripheral nerve allografts [97,121]. To shed light on the issue, Mackinnon et al. [57] investigated the nerve allograft response between closely and distantly related inbred strains of rats using both histology and the ^{51}Cr release cytotoxicity assay to assess antigen recognition. Briefly, the ^{51}Cr assay is used to assess the cytotoxic ability of a sensitized recipient animal. Immunocompetent animals are sensitized

to an antigen (a peripheral nerve allograft) thus stimulating the production of cytotoxic elements which express antigen binding receptors specific for the antigen which stimulated their development. When the cytotoxic cells meet this antigen again (this time carrying a chromium 51 radioactive tag) the cells proliferate and lyse the antigen carrying cell thus releasing the chromium to be excreted in the urine of the animal where it can be easily measured [57]. In Mackinnon's experimental model, Lewis rats received 4.0 cm sections of Fisher, Buffalo, and ACI rat sciatic nerves [57]. Lewis and Fisher rats are known to differ only with respect to minor transplantation antigens whereas Lewis, Buffalo and ACI rats differ at major histocompatability loci [74]. Results of the ^{51}Cr assay showed sensitization with Buffalo and ACI nerve segments as early as day eight, whereas in the matched Fisher-Lewis grafts sensitization did not occur until day eighty. By contrast, skin allografts induced sensitization by day ten in all three strains. By day five, histological exam revealed the same dense cellular infiltrate in each group even when the histocapability was matched for major transplant antigens. The response in the latter group was, however, characterized as slower [57]. Although an immune reaction was seen histologically as early as day five in the Lewis-Fisher model none was seen by ^{51}Cr assay until day eighty. The ^{51}Cr assay depends on the presence of cytotoxicity and as such measures the functionally

significant components of the immune reaction. Since the assay has been previously shown effective in measuring sensitization in strains differing with respect only to minor transplantation antigens, Mackinnon et al. [57] concluded that these cells in the immune response though present histologically are less immunocompetent than cells involved in the response between strains with major antigenic differences. If valid, these findings would cast doubt on the reliability of histology to quantitate the immune response.

In a recent study Mackinnon et al. [60] investigated the actual degree of regeneration that would occur across a nerve allograft actually implanted with microsurgical technique. They employed the same inbred rat strains as used in the previous immunological study judging nerve regeneration at five months by histological (axonal counts) and electrophysiological (nerve conduction velocity) measurements. The authors noted good regeneration in autograft and matched allograft groups and poor results in non-matched allografts. Mackinnon et al. concluded that these findings correlate well with both the histoincompatibility status of the donor and host animals and with the author's previous immunological study [57,60]. These findings do not correlate with those of Zalewski et al. [118,121], but do agree in respect to immunogenicity of minor antigenic differences with the findings of Singh [95,97]. Again, as in most the studies

in the literature, no attempt was made to describe experimental results with respect to functional return. Almquist [4] has shown the total lack of correlation between clinical results and nerve conduction velocity in man, thus, limiting the value of extrapolating electrical measurements obtained from experimental animals to presumed clinical application.

In a separate study, Mackinnon et al. [59] addressed pretreatment methods used to decrease antigenicity of peripheral nerve allografts by using the same ^{51}Cr immunologic assay to determine quantitatively the nerve allograft response. Mackinnon looked at the effects of low and high dose irradiation, predegeneration, freezing with a cryoprotectant agent, lyophilization, and combination pretreatments in rats known to differ at the major histocompatibility locus [59,74]. They described these methods as having been used previously in clinical and experimental settings. As in previous work the donor nerves were not sutured in place but rather inlaid between the gluteal muscle fibers of the recipient rat [57]. Results showed that when the nerve received a high dose of irradiation or was lyophilized the immunological response did not differ from control autografts. They conclude that lyophilization and high dose irradiation can modify the nerve allograft in such a way as to render it less immunogenic while other pretreatment methods fail to do so [59]. It is interesting that Mackinnon chose to use a

cryoprotectant agent in her cryopreservation study while Singh [97,94,95] and Zalewski et al. [118] did not. It would have been useful to present data involving cryopreserved allografts prepared without a cryoprotectant agent. Verhoog and Van Bekkum had shown, in 1971, that high dose irradiation and freezing without a cryoprotectant kills cells, thus, rendering them non-antigenic [109]. From this one might conclude that these grafts frozen without cryoprotection would also prove less immunogenic by the ^{51}Cr assay. It is obvious that lyophilization kills cells, thus, these three pretreatment methods are similar in that the allograft when transplanted is dead.Mackinnon's conclusions are therefore no different from those of Verhoog and Van Bekkum [109] thirteen years earlier. Complex immunologic assays addressing the immunogenicity of grafted segments have little to do with judging their functional ability to serve as more than a lifeless conduit for axonal regeneration. In an assessment of regeneration across lyophilized and irradiated allografts, Mackinnon et al. using previously described parameters [60] described regeneration as intermediate between that occurring across a fresh, untreated nerve allograft and that occurring across control autografts [58]. Pollard [80] had shown previously that results with autografts were superior to allografts as a result of the autografts ability to conduct a column of Schwann cells and regenerating axons to the periphery.

Mackinnon has shown through immunological studies that killing the cells of an allograft decreased its antigenicity [57,59] and in so doing she supports the results of previous investigations [13,14,29,64,65,85]. She failed, however, to prove that this has functional significance with respect to augumenting axonal regeneration. Ducker and Hayes, in 1970, had reported that allografts treated with such cytotoxic methods were only successful when used in lengths less than 4.0 cm [29].

In order to exceed these graft length limits and to more closely mimic the autograft response research needs to be directed toward preserving viable allogenic graft cells to potentiate axonal regeneration while decreasing the host response to these cells.

In a series of papers dedicated to the role of the immunosuppressive agent cyclosporin A in the prevention of the rejection of peripheral nerve and Schwann cells in allografts, Zalewski, Gulati, and Silvers [115,116,119,120] reported their results from work done at NIH in the early 1980's. They showed that nerve and Schwann cells survived in allografts in rats treated with cyclosporin A [116]. In separate work they demonstrated that host nerve axons regenerate through long nerve allografts and become insheathed by allogeneic cells if the host is immunosuppressed by rendering it tolerant at birth to the transplantation antigens of the nerve donor [121]. By innoculating lymphoid cells from specifically sensitized

isogeneic donors into tolerant rats, thus, abolishing immunologic tolerance, the authors precipitated rejection of the allogeneic Schwann cells as well as the regenerating host axons in the allograft [117]. In a recent paper, Zalewski, Silvers, and Gulati [122] investigated the extent of renewed axonal growth into the connective tissue remnants of rejected nerve allografts. Their goal was to investigate whether nerve segments in connective tissue continuity with the host nerve provides a framework for regeneration as in Mackinnon's and other models of pretreated allografts. The authors found, however, that the host axons only regrow into the most proximal part of the allograft. They conclude that viable allogeneic cells such as Schwann cells and fibroblasts together with the connective tissue matrix provide the best way to potentiate axonal regeneration through long allografts [122]. These findings support the need for establishing a reproducible and efficient way to store peripheral nerve allografts without killing allogeneic cells.

C. Cryopreservation

Controversy exists in the literature as to the role of cryopreservation in peripheral nerve allografting. Singh et al. [94,95,97] demonstrated that cryopreserved allografts could conduct regenerating axons. Zalewski, Gulati, and Silvers [118] failed in their work to support Singh's conclusions. Verhoog and Van Beckkum [109] showed

that freezing without a cryoprotectant agent as in Singh and Zalewski's work killed cells rendering then non-antigenic. Mackinnon [59] disclosed indirectly by ^{51}Cr immunological assay that peripheral nerve cells could survive if treated with cryoprotectant agents. Zalewski, Gulati and Silvers [122], in their most recent work, conclude that these viable allogeneic cells such as Schwann cells and fibroblasts are needed to provide the best way to potentiate axonal regeneration through long allografts. It is the goal of this research to explore optimal methods for preserving cell viability during cryopreservation and to employ cryopreserved grafts in a physiological manner in an effort to compare their effectiveness in potentiating axonal regeneration to that of nonpreserved autografts.

Scientists have been exploring the effects of low temperature on biological and experimental models for centuries and have long contemplated the storage of living cells, tissues, organs and, even individuals for various medical and non-medical reasons. The concept of a cryoprotective agent is, however, a much more recent development. Keith [128], in 1913, reporting in Science was among the first to describe improved survival of microrganisms when frozen in milk, sugar, or glycerol solutions over those frozen in water or saline. Luyet and Hodapp [130] described improvement in frozen frog spermatazoa survival after pretreatment with a sucrose

solution. Other authors observed the protective action of sugars in erythrocytes subjected to rapid freezing. These early authors attributed the advantages of these treatment methods to non-specific effects such as providing channels between ice crystals in which cells might escape from being crushed [127]. In 1949, Polge, Smith, and Parkes [131], following studies involving the protection of spermatazoa and erythrocytes by glycerol, were the first to recognize and appreciate that specific compounds provided direct protection from some undefined parameter of freezing. Lovelock and Bishop [129] discovered later that Dimethylsulfoxide (DMSO) could effectively protect spermatazoa and erythrocytes from freezing injury and could serve almost universally as a protective compound. Doebbler [28] investigating the molecular structure which confers cryoprotective activity upon a substance found that multiple hydrogen-bonding sites per molecule markedly magnify an agent's protective capacity and that DMSO is an excellent hydrogen bond acceptor.

Two main theories have surfaced to explain the protective mechanism of DMSO and other cryoprotectant agents. The first holds that by lowering the freezing point of solution DMSO prevents damaging ice crystal formation within cells. Intracellular ice formation has been shown to alter the spatial configuration of important intracellular molecules such as catalytic enzymes. The second theory maintains that DMSO acts by lowering the

temperature at which ice will separate out of solution and in so separating cause an increase in the concentration of the electrolytes in solution to levels which are toxic to the cells. The increased extracellular concentration results in an osmotic gradient with subsequent flow of water out of the cells leading to harmful concentrations of electrolytes [107] within the cells as well as to dehydration and consequent denaturation of cell proteins and destruction of cell enzymes and their enzymes patterns [52].

In an experiment designed to determine which of these two factors was the most important, Farrant [32] added solutions during cooling to prevent the concentrations of solutes in the liquid phase from rising above normal values, both in the presence and absence of ice crystal formation. In order to prevent ice crystal formation at temperatures as low as minus 80^{o} C, some of the solutions included DMSO in very high concentrations. Farrant found each method either with or without ice crystal formation to protect mamallian muscle tissue from cryo-damage and, thus, concluded that the increase in electrolyte concentrations to toxic levels was more important and its prevention was the protective action offered by DMSO and other agents [32].

Addressing Farrant's results, Karow, Carrier, and Holland [47] demonstrated that exceedingly high concentrations of DMSO needed to prevent ice crystal

formation were unnecessary and toxic in rat heart preparations when used in concentrations greater than 25 percent DMSO.

In a study involving the role of DMSO as a cryoprotective agent for peripheral nerve tissue, Lenz et al. [52] applied a silver cryoprobe to the sciatic nerve of rabbits in vivo, one-half of which had been pretreated with a 10 percent DMSO solution. By macroscopic observation, electrophysiological measurements, and histological examination, Lenz concluded that DMSO was effective in reducing the damage caused by the freezing process in peripheral nerves which had been pretreated with a 10 percent solution.

In later work, Farrant et al. [33] investigated the role of a two-step cooling procedure first described by Luyet and Keane in 1955 in examining factors influencing cell survival following freezing and thawing of Chinese hamster lung fibroblasts. Farrant, using a 5 percent DMSO solution, measured survival by a cell colony assay and examined the ultrastructure of the cells to determine the degree of cellular shrinkage and the location of intracellular ice. Farrant found that cellular shrinkage induced by holding at -25^{o} C to -35^{o} C for ten minutes correlates with protection from injury. Alternatively, rapid cooling without holding at sub-zero temperature induced intracellular ice formation. In thawing rate experiments, Farrant et al. found an increased sensitivity

to injury as a result of slow thawing which was most marked in the case of rapidly cooled cells and, thus, linked such injury to intracellular ice formation. In conclusion, Farrant et al. suggests that injury occurs during rewarming and is osmotic in nature.

In a recent article, Tomford et al. [106,107] reported the results of investigations on the effects of DMSO and glycerol on chondrocytes as well as the ability of this cell to function normally after freezing. The authors' found that at 4^{o} C neither cryoprotectant produced toxic effects unless their concentration was greater than 12 percent or exposure time greater than ninety minutes. Using the previously established two-step cooling technique, they combined various pre-maximal freezing temperatures with various exposure times. The optimal freezing technique for cells previously exposed to DMSO as judged by trypan blue dye exclusion was when chondrocytes were cooled to -40^{o} C for five minutes before flash-freeze to -80^{o} C. Survival in cells pretreated with DMSO approximated 95 percent while control survival in untreated cells was 60 percent. To evaluate the metabolic products of fresh and frozen chondrocytes, the authors placed surviving cells in culture and found that cells remaining in culture for seventy-two hours after thawing produced aggregated proteoglycans similar to those made by fresh chondrocytes.

Tomford et al.'s dog chondrocyte results are similar to

those of Farrant [33] working with Chinese hamster lung fibroblasts. In both systems a first stage cool to -35^{o} C to -40^{o}C before deep freeze coupled with a rapid thaw produced good functional results [33,106,107].

The established two-step freezing technique will be used and the temperature and thawing parameters established by Tomford and Farrant tested in our experimental model of nerve cryopreservation of peripheral nerve autografts in the rat.

D. Animal Model

A superlative regenerative capacity in peripheral nerve injury has been suggested in the rat which Mackinnon et al. [123] claims to undermine the validity of studies investigating factors used to evaluate nerve regeneration. Mackinnon further states that controversey exists over the appropriateness of the rat as an experimental model for studying peripheral nerve injury, repair, and regeneration. The authors cite two sources to back their claim. The first, a 1964 study by Kline, Hayes, and Morse [50] comparing the response of species to peripheral nerve injury and, the second, a 1982 letter to the editor which paraphrased Kline et al.'s results [37].

In their study, Kline et al. [50] carried out neural crush and severance with primary suture repair in a series of dogs, monkeys, baboons and chimpanzees. They compared results by histological exam, electrophysiological studies,

and axonal counts. The authors conclude that differences exist in species in response to peripheral nerve injury, specifically with respect to connective tissue proliferation in phylogenetically higher animals. However, in each comparison performed at two to six week intervals, the authors presented data from only one animal in each group. Futher reported differences were concluded by subjective criteria alone. In addition, despite these apparent differences of species in the pattern of regeneration, differences in axonal counts were never statistically significant among the four species in either the crush or severence with primary suture repair model. Obviously when experimental results are to be applied to humans, it is best to utilize higher primates as models, but it would not seem warranted by the above conclusions alone to abandon the rat model which offers an inexpensive and relatively unlimited source of mammalian nervous tissue which even at the electron microscopic level is indistinguishable from human peripheral nerve.

In a stated recent attempt to lend perspective to the significance of previous studies and perhaps to serve as a control for future studies, Mackinnon, Hudson, and Hunter [123] investigated the degree of spontaneous regeneration that occurs in the sciatic nerve of rats five months after complete resection. Histological examination in thirty adult Lewis rats five months after sciatic nerve excision revealed an average proximal to distal regeneration of 23.7

mm ± 6.4 mm. Histologic sections taken at 1.0 cm intervals along the regenerated nerve segments revealed evidence of compartmentalization and "minifascicle" formation.

The findings of this study are interesting when compared to contradictory results from other independent studies in the literature involving spontaneous bridging of peripheral nerve gaps. Lundberg et al. [54] comparing the anatomical and functional regeneration of a transsected sciatic nerve following regrowth from its proximal stump through either preformed empty mesothelial chambers or autologous nerve grafts found little or no regeneration when a 1.0 cm gap between the nerve stumps was left empty in Sprague-Dawley rats.

Bucko and Steinmuller [14] studying reinnervation of tail muscles after orthotopic grafting of peripheral nerve segments in inbred rats found in their control series of nineteen animals that none were able to spontaneously bridge a 3.0 cm gap to reinnervate tail muscles during an eleven month period of observation.

Ducker and Hayes [29] investigating autograft limitations in dogs and chimpanzees found each incapable of spontaneously bridging a control gap of 2.0 cm. Contrary to their prior conclusions, it seems impractical to extrapolate Mackinnon's results [123] to serve as controls for unrelated studies of peripheral nerve regeneration as evidenced by the aformentioned gap studies. Many known and unknown factors are involved in the regeneration of

peripheral nerve tissue in various experimental systems. It would, therefore, seem most efficacious to eliminate unnecessary variables by utilizing gap animals unique to each experimental model as controls against spontaneous bridging of peripheral nerve defects and to quantitate specific regenerative capacity.

E. Physiology of Repair

Many studies have been performed and various techniques devised to address the need to optimize the suture line and, thus, potentiate regeneration across proximal and distal stumps in peripheral nerve grafting. Studies have shown that even in cases of peripheral nerve repair under the most optimal of conditions that a small zone of fibrosis or scar tissue developed between the repaired nerve ends which needed to be tranversed by regenerating axons. This phenomena is especially important in the case of free nerve grafting when not one, but two suture lines need be crossed. Berger and Millese [11] as a result of their experience in animal experiments and clinical practice have shown that regenerating axons can cross two optimal suture lines more easily than one poor one. Through their work, they have defined conditions to optimize the suture line as follows. Firstly, that no gap be present between the proximal and distal stump after repair. Secondly, that there be only minimal tissue proliferation at the site of anastomosis. And thirdly,

that there be optimal coaptation of major fasciculi or groups of minor fasciculi between the graft and host tissue. From their large series of experimental and clinical cases, Millesi and Berger [71] have further identified the most important negative factor influencing the results of nerve repair carried out under the aforementioned optimal conditions as tension at the suture site. Results and recovery rate are statistically better if tension is avoided.

The advent of the operating microscope coupled with fine surgical instruments has afforded modern surgeons the ability to place extremely fine #10-0 and #11-0 epineural and perineural sutures. This minimizes surgical trauma to the nerve and permits precise anatomical alignment of severed stumps. However, suture material acting as a foreign body may evoke fibrous tissue formation which Berger and Millesi [11,71] have shown to adversely affect axonal proliferation. In an attempt to eliminate this foreign body reaction, some authors [29,118,121,120] have performed neuroplasty utilizing fibrin gluing or clotting techniques which mimic the natural coagulation process at the anastomotic site. In an attempt to compare the efficacy of these two methods of neuroplasty, Becker, Gueuning, and Graff [9] performed peripheral nerve anastomoses utilizing the rat sciatic nerve as a model using either epineural sutures or a fibrinogen adhesive technique. Using radiolabelling of the metabolically

active acid-soluble phosphate fractions of nerve and muscle and comparing gastrocnemius and soleus muscle weight in a number of experimental situations Becker et al. [9] showed that labelling of the sutured and glued nerves was not significantly different with the return to control values proceeding at the same rate. In addition at 60 and 150 days there was no significant difference in innervated muscle weights between the suture and fibrin glue groups. Both repair methods were shown to be equally inferior to the spontaneous recovery which occurs after simple nerve crush injury.

In an interesting study designed to determine the effect of nerve graft polarity on nerve regeneration and function, Stromberg, Vlastou, and Earle [103] found by direct conduction velocity and amplitude studies that nerve graft function was statistically independent of nerve graft polarity. This finding, though not directly related to the current study, decreases the possibility that a reversal of orientation during either storage or reimplantation of the autograft would affect our experimental model and the interpretation of results.

deMedinaceli, Wyatt, and Freed [25,26,34] working in response to the resultant gap which results from neuroplasty using either nerve suturing or gluing with blood or blood protein have devised a highly complex technique designed to address some of the conditions as defined by Berger and Millesi [11] which affect the

regeneration of axons at sites of anastomosis. Their technique considered seventeen factors of which the major ones were proper alignment of the stumps, sharpness of the cut and prevention of physical and chemical damage to the severed nerve. In their procedure, which they term nerve reconnection, the transsected nerve stumps are sutured to a rubber support in an overlapping fashion. The nerve is bathed in a modified Ringer's solution to prevent the interior of the nerve fiber from losing its chemical and ionic balance. Next, the nerve is cooled and the ends trimmed with a vibrating razor blade to assure a sharp transsection of the axons with minimal resultant crush injury. The tension on the rubber support is then released until it pushes the nerve ends together with just enough pressure to hold them in place without distorting them. The temperature is then raised and the ends of the rubber support are sutured together. They report results in the rat based on functional criteria (which will be further explained in the next section) in reconnection technique which were 66 percent better than that of animals treated by conventional microscopic technique [25], and suggest the use of this method to improve the return of function after peripheral nerve injury in humans. Unfortunately, deMedinaceli,s technique is a long and highly complex procedure which requires extensive dissection, disruption of neural circulation, application of freezing and nonphysiological solutions, removal of a portion of the

nerve itself, insertion of a foreign body which requires a second operation for removal and a surgically ideal clinical situation of transverse nerve transsection without associated local injury. The procedure's clinical usefulness is limited by tension at the repair site; the very problem it attempts to address. In addition, no attempt has been made to use nerve reconnection in the case of segmental loss requiring an autograft or allograft.

In light of these limitations and with respect to equivocal findings with respect to fibrin gluing and suturing, we have chosen to employ conventional microsurgical suturing technique in our experimental model.

F. Methods for Assessing Regeneration Following Grafting

1. Functional Recovery

In the evaluation of the relative efficacy of various types of peripheral nerve grafting procedures or pretreatment techniques, it is clear that the most important criteria for judging recovery in meaningful terms would be return of function to the reinnervated limb. Although easily assessed in man, functional recovery is difficult to measure in experimental animals. In 1982, deMedinaceli, Freed, and Wyatt [24], realizing that no widely accepted quantitative method existed to evaluate the functional effects of peripheral nerve damage in animals, devised a method for evaluating sciatic nerve damage from measurements of the foot prints of the hind foot of walking rats. From the walking tracks they measured four variables to which they compared the operated to control leg and converted their findings into percent deficits which they averaged to obtain what they termed a "sciatic functional index" (SFI).

The method of deMedinaceli et al. involves testing animals in a confined walkway with a dark shelter at one end. A piece of unexposed x-ray film is placed along the floor of the corridor and the animals' hind feet are dipped

into a developing fluid. As the animal walks down the corridor, paw prints appear immediately on the film. Three or four prints from each foot typically appear on film from which the authors measured four variables. The first measured variable is the distance from the tip of the experimental foot to the tip of the following contralateral toes. This variable measures the capacity of the experimental leg to support the animal's weight. The second variable measured the length of a foot print (PL). This length is short in normal animals which walk only on their toes and longer in animals with neural deficits. The third variable measured the linear distance between the first and fifth toes and defined this variable as the toe spread (TS). The final variable measured the toe spread between intermediary toes as the linear distance between the second and fourth toes (IT). The latter two variables served as an indicator of the condition of the peroneal branch of the sciatic nerve. From these variables, deMedinaceli et al. devised an empiric formula based on the assumption that all four variables were equally important. The authors tested their index in animals receiving a sham operation, a Ringer's injection into the sciatic nerve trunk, a neural crush injury, and transsection with immediate suture repair. They found the speed of functional recuperation observed by the SFI to be consistent with established values for other methods [27,39]. Recovery was observed in the injected nerve by

the second day, whereas the sham operation did not effect the SFI from normal values. The crush injury nerves recovered preoperative values by the thirty-second day postoperatively while the severed and repaired nerve showed no statistically significant recovery at thirty-two days. The authors concluded that the sciatic functional index provides a precise and reliable assessment of rat sciatic nerve recovery [24].

deMedinaceli's results apply nicely to the situation of minor nerve injury or crush injury when the nerve architecture is injured but maintains its support architecture. They, unfortunately, did not report data from their group with repaired transsected nerve injuries past thirty-two days postoperatively. The authors further used many "adjustment factors" and extrapolation formulas to account for animals who suffered self mutilation or whose values fell outside of established guidelines. It is possible that the SFI does not apply readily to situations involving major nerve injury due to the introduction of more variables such as flexion contracture or self-mutilation. We will investigate this in our study by applying the SFI to animals which have received sciatic nerve autografts.

2. Electrophysiological Studies

Secondary to the inherent difficulties involved with neurological examination of experimental animals involving

its lack of objectivity and its potential to lead to biased results, many authors have used direct electrophysiological measurement of the nerve graft segment in the form of evoked muscle action potentials and in vivo nerve conduction velocity measurements to draw functional conclusions from their grafting experiments [14,29,56,60, 81,82,83,94,95,96,97]. Although some authors have found progressive recovery of compound muscle action potential in experimental animals, they have had difficulty describing results in objective terms referring to wave forms as relatively normal, though of reduced size [81]. Because of these problems, difficulties eliminating cross talk, and troubles with the elimination of other tissues that can conduct eletrical impulses, most of the investigators [29,56,81,94] have turned to nerve conduction velocities to quantitate recovery. In these experiments, the nerve graft is exposed and the sciatic nerve stimulated through paired silver wire electrodes placed beneath the nerves. The maximum conduction velocities of the graft are determined from the latency of the proximal and distal stimulating points to the muscle and the conduction distance. MacKinnon [56,60] and Pollard [81,82,83] used conduction times to support their conclusions while Singh [94,95,96,97] used conduction velocity results to divide his animals into four EMG grades of decreasing velocities. Unfortunately, Almquist and Eeg-Olofsson [4], in their study of nineteen patients, have shown in their 1970

article relating nerve conduction velocities to two-point discrimination in sutured nerves that there is no correlation between the clinical results as measured by the functional two-point discrimination test and the nerve conduction velocity. Therefore, the maximum conduction velocities studied had no functional significance as purported by the aforementioned investigators [14,29,56,60, 81,82,83,94,95,96,97] and their findings should not be extrapolated to possible human application. For these reasons, EMG studies will not be performed in our experimental model.

3. Sensory Testing

Although sensory testing is an excellent marker in humans for functional return, it is subjective and unreliable in animals such as the rat. Even a positive or negative response to painful stimuli is difficult to interpret in light of cutaneous reinnervation following peripheral nerve injury and repair, secondary to the expansion of the functional distribution of intact neighboring fibers of uninvolved nerves. For these reasons, investigators have not used sensory return as a criteria to judge their results in peripheral nerve allograft experiments. Devor, Schonfeld, Seltzer, and Wall [27] have reported recently on their work involving cutaneous reinnervation following peripheral nerve injury which could in an altered form be used to make conclusions

concerning the recovery of sensation in the distribution of the sciatic nerve in rats. Cahal [17] had shown in 1928 that the mechanism of return of sensate function involves movements of two interacting populations of axons: regenerating fibers from the severed nerve and sprouts emitted by intact fibers in the skin surrounding the denervated zone. Devor et al. [27] investigated these movements behaviorally and electrophysiologically in rats with experimentally induced crush injuries or permanent nerve section of rat sciatic nerve. The foot in the rat receives its cutaneous sensory innervation along the saphenous nerve (an extension of the femoral nerve) and the sciatic nerve. Devor et al. determined the normal distribution of each nerve by cutting the other and mapping the regions of sensory loss and remaining sensibility. They found the saphenous nerve to innervate only the dorsal aspect of the medial toes. By studying changes after crush of the sciatic nerve with the saphenous nerve intact or ligated, they further show that the early phase of sensibility recovery is attributable to invasion by branches of the intact saphenous nerve (four days) resulting in expansion of its distribution and the later phase to regeneration of the sciatic nerve itself (twenty days). Detailed observations were made on rats in which the sciatic nerve was severed and regeneration prevented. The authors found that the area of responsive skin began to increase within four days as before, but after fifteen to

thirty days stopped short without returning to the lateral most toes (toes five, four, and most of three). Accompanying this functional expansion, the authors discovered expanded saphenous nerve receptive fields consisting of high threshold mechanoreceptors. With return of function of the sciatic nerve, the expanded distribution of the saphenous nerve went back to its original boundaries. The authors concluded that cutaneous reinnervation begins with collateral expansion of high threshold afferents from intact neighboring nerves which are later replaced upon regeneration of the original nerve. Devor et al. did not see or investigate the possible application of their findings to the nerve graft model. Based on their findings that the saphenous nerve does not reach to reinnervate the lateral toes, we can show that in the nerve grafted animals return of sensibility to the lateral foot is solely the result of reinnervation via the sciatic nerve graft. In fact, we can follow lateral sensation return in experimental animals as an early index of sciatic functional sensory return giving us an objective test of functional recovery.

4. Hydroxyproline Assay

The hydroxyproline content of animal tissue is thought to be confined almost exclusively to the connective tissue scleroproteins, collagen and elastin. Hydroxyproline concentration has, as a result of this restricted

distribution, been used as an indicator of both the presence and the metabolism of collagen. Historically, the most commonly used quantitative assay for hydroxyproline was that of Neuman and Logan [73]. Unfortunately, their assay was highly susceptible to interference from other amino acids in the hydroxyproline containing samples and not sensitive to low levels of hydroxyproline in tissue samples. In 1961, Woessner [112] addressed these problems in developing a method for the quantitative determination of hydroxyproline concentration in biological materials containing as little as one part of hydroxyproline in four thousand parts of amino acid. Reporting at the Orthopaedic Research Society in 1986, MacDonald et al. [56] described their findings involving hydroxyproline quantitation in normal muscle and denervated muscle after immediate and delayed nerve repair using the Woessner assay. Intramuscular hydroxyproline levels are known to be a direct indication of collagen content. Acetylcholinesterase and elastin are the only muscle proteins which contain significant amounts of hydroxyproline, less than one percent by weight. In studies undertaken to determine whether overall increases in intramuscular collagen follow denervation and reinnervation, MacDonald et al. found that functionally successful immediate nerve repair results in a doubling of intramuscular hydroxyproline content in three months. Muscles from nonfunctional delayed repairs show a five-fold

increase over normal hydroxyproline levels and a two-fold increase over cases involving immediate repair. In all cases, there was correlation between function and collagen content, with increase in fibrosis indicating poorer functional recovery. Our study will incorporate Macdonald et al.'s findings. By assessing tibialis anterior muscles from experimental and control legs in each group we will use the hydroxyproline concentration as another method to judge functional recovery.

5. Axonal Counts

Traditionally axonal counting methods have been employed to assess the degree or efficacy of axonal transmission through nerve graft segments from the proximal to distal stump. Well myelinating fibers have been correlated with electrophysiological evidence of regeneration. Many histological quantifications have been made from results of axonal counts from regions proximal, within, and distal to grafted segments. In addition to the objective number of myelinated nerve fibers, fiber diameters and percentage of neural tissue have been described. In the case of allografted tissue, comments have also been made concerning inflammatory or graft rejection phenomena. While axonal counts are useful in assessing axonal transmission, they do not account for possibilities such as a single proximal axon being represented by more than one axon in the distal

segment. More importantly, no direct functional correlation may be drawn from results of axonal counts. Axonal counts as an indicator of axonal ingrowth can however be inversly correlated with graft rejection or death of graft Schwann cells. Realizing these limitations and their application, we will conduct axonal count studies, the results of which will be applied to more functionally objective criteria.

G. Conclusion to Review of the Literature

Finally, in assessing the relative merits of different types of grafting procedures, care must be taken to evaluate the extent of recovery in functionally meaningful terms. Some authors have devised experimental models in which they performed only proximal anastomoses [118,120,122]. Others have placed 4.0 to 5.0 cm grafts into 0.5 cm deficits requiring graft segments to be looped around within the experimental animal's leg [61,118,122]. Still others have implanted nerve segments in ectopic regions [13,19]. Clearly, these models do not present functionally significant data. Most investigators have used some form of axonal counting and/or electromyographic study alone to draw conclusions from their results [14,29,57,59,75,83,95]. The dangers of extrapolating data to the human model have been previously discussed. In this study, we shall approach interpretation of our data from a multifaceted analysis combining traditional methods with those newly

devised. We will combine pure functional studies with sensory, histological, anatomical, and biochemical analysis in an attempt to maximize the validity of our experimental results and in such potentiate the comparison of experimental results to possible clinical usefulness while realizing the limitations of extrapolation.

III. Material and Methods

A. Animal Model

Adult male Sprague-Dawley rats weighing 300 to 320 gm were purchased from Cam Breeding Laboratories (Wayne, New Jersey). All animals were maintained in plastic lined cages in a central animal care facility (FMB-05) and received water and rat chow ad libitum. The animals' daily diurnal cycle was set at twelve hours light followed by twelve hours darkness. Three experimental groups were used. The first control grafted group (n=11) received a 2.0 cm fresh autograft. The second cryopreserved grafted group (n=12) received a 2.0 cm cryopreserved graft. The third gap nongrafted group (n=13) was left with a 2.0 cm deficit in one sciatic nerve.

B. Surgical Procedures

Autograft techniques were chosen in an attempt to minimize the role of histoincompatibility in this model. Even inbred rat strains have some minor histocompatibility differences which could adversely affect our results [126]. The right or left sciatic nerve of three groups of fifteen animals was exposed through a gluteal muscle splitting incision. The animals were unselected and randomly assigned to treatment groups. Only one side of each animal was operated upon. The other rear leg served

as an internal control for each animal. The sciatic nerve was then exposed in its length from the sciatic notch proximally to the distal muscular branches in the region of the popliteal fossa. A 2.0 cm segment of sciatic nerve beginning proximally 0.5 cm from the sciatic notch was resected. In the eleven animals compromising the control group, the 2.0 cm sciatic segment was left with its vascular pedicle intact and each of its ends were tacked down to local muscle fascia using microsurgical technique (operating microscope JK-Hoppl Company, Model No. 029001) with one epineural suture of #10-0 nylon (Dermalon, American Cyanamide, Pearl River, New York) to maintain its length.

In the twelve animals comprising the cryopreserved group, the 2.0 cm segment was first suspended using microsurgical technique to a micro-clamp with two #10-0 sutures and then placed into sterile tubes containing culture media that consisted of 90 percent DulBeccos modified Eagle's medium, supplemented with 10 percent fetal calf serum and containing 10 percent sterile DMSO as an anticrystallizing cryopreservation agent. After four to five minutes in this medium, the micro-clip containing the nerve was removed and placed into a separate, sterile freezing vial (NUNC 4.5 ml Cryotube, West Germany). The vial was immersed for 300 seconds in an acetone bath maintained at -40^{o} C. The temperature of the bath was constantly monitored by a cryoprobe. The vials were then

stored at -80° C in a Revco freezer for three days. In preparation for reimplantation, the nerve segments were rapidly thawed in lactated Ringer's at 37° C for three minutes.

In the thirteen animals comprising the gap group, the 2.0 cm sciatic segment was removed and used for histological studies of normal axon counts. The values obtained, expressed in terms of axonal density, were compared with counts obtained from similar sections cut through the graft segment of the fresh and frozen nerve graft segments at the time of sacrifice. The two ends of the resultant gap were sutured to fascia with #10-0 suture utilizing microsurgical technique at a gap distance of 2.0 cm between proximal and distal segments. In all groups, the gluteal muscle was approximated and the skin closed with #3-0 Dexon.

At seventy-two hours, the control group underwent a second operation at which, using microsurgical technique, an epineural repair was carried out using four sutures of #10-0 nylon. Physiological realignment of the proximal and distal segments was achieved by marking the stumps at the time of the original procedure with blue dye (methylene blue chloride, H.J. Conn, Geneva, New York) and by realigning surface vessels. The vascular pedicle was then separated. The repair was the same in the cryopreserved group. The gap group received a sham operation, at which time the sciatic nerve was exposed. Again, the gluteal

incision was approximated and skin closed using #3-0 Dexon. All operations were performed using aseptic technique in the Orthopaedic Surgery Operating Laboratory. All animals wounds were treated with Povidine Prep Solution (ACU-dyne, Fairfield, Conn.) prior to final closure. The anesthetic used was pentabarbital sodium (30 to 40 mg/kg, Abbott Laboratories, Quebec) administered intraperitoneally.

C. Functional Testing

The animals were observed two to three times a week to identify any wound or ulcer complications. In addition, the animals were followed by Serial Gait Testing preoperatively and at two-week intervals throughout the course of the experiment. The animals' gait testing was conducted in a confined walkway fifteen inches wide by fifty inches long with a dark shelter and peanut butter reward at the far end. This design is a modification of that described by deMedinaceli [24]. After one or two conditional trials in which the rats stopped to explore the corridor and later in the course without any trials, the rats walked steadily to the shelter and reward at the end of the darkened corridor. A piece of unexposed x-ray film (Kodak X omat TL Film, Eastman Kodak, Rochester, New York) cut to the proper dimensions was placed on the corridor floor. The rats were then held in one hand while applying developer (Dektol, nondiluted, Eastman Kodak, Rochester,

New York) to the animals hind feet with a dampened sponge. The animal was placed at the entrance to the corridor and walked steadily along the x-ray film causing paw prints to appear immediately on the film.

Three or four prints of each foot appeared on the length of the film used. Three variables were measured on the control and operated paw prints. The toe spread (the linear distance from the center of the print of the first toe to the center of the print of the fifth toe) measurement on the experimental side was expressed as a percentage of the corresponding value measured on the control side. The other parameters measured were the print length (the length of one foot print) and the distance between the intermediary toes (the linear distance between the centers of the second and fourth toes). All values were measured to the nearest millimeter (see Tables 1-3). To test the reproducibility of the index, forty-five tracks selected at random were measured by two observers who were not previously trained. Their results were compared with those obtained for the same forty-five tracks by the principal observer.

At the time of sacrifice while under anesthesia, each animal's hind foot passive range of motion was assessed in the normal and operative leg and degree of extension measured with a goniometer. To test the reproducibility of the measurements, four nonselected animals in each group were measured by an observer not previously trained. His

results were compared with those obtained by the principal observer.

D. Sensory Testing

The rats' feet were examined preoperatively and at close intervals after surgery. At two week intervals concurrent with gait testing experiments sensory examinations were performed. In these tests, the animal was minimally restrained, the leg was grasped gently and six predetermined sites on each foot were examined for response to pressure or pinch stimuli applied to the skin using an Addison's forcep. Each site was graded on a scale from zero to three indicating: 0) no response even to strong grasping of the skin, 1) vocalization or withdrawal, but only upon strong pinching, 2) vocalization or withdrawal with moderate pressure, but less reliably or more sluggishly than with the same stimulus in the mirror spot on the contralateral nonoperative foot, and 3) normal response defined as equal to that obtained in the same spot on the contralateral leg. In accordance with this classification, the animals' reactions were graded and reported on a scale from zero to eighteen. At the time of sacrifice, animals while lightly anesthetized were again tested as above, but now in a highly controlled experimental conditions. To test the reproducibility of the index, fifteen animals selected at random were tested by an observer with no knowledge of the experimental

design. His results were compared with those obtained by the principal investigator. These tests provided reliable and reproducible results.

E. Hydroxyproline Assay

Following re-exploration and harvest of the nerve graft segment, attention was directed to the muscles of the anterior compartment of the rats' experimental and control legs. A midline incision was made from the superior aspect of the patella to the first web space medial to the great toe, and the tibialis anterior and the extensor digitorum longus muscles were identified and harvested at their origins taking care to divide their tendon insertions at the level of the inferior extensor retinaculum. The muscles were weighed (Satorius Analytical Balance Model No. 2603, West Germany) to the nearest .00005 gram on an analytical balance and frozen at -10^{o} C in a sterile cryotube for later analysis.

In each of the three experimental groups studied intramuscular hydroxyproline in each of the rats tibialis anterior muscles was quantitated at three months following the initial procedure by the procedure of Woessner [112]. Muscles to be assayed for hydroxyproline were first thawed and then stripped of tendon and surface connective tissue. Two 30 to 50 mg pieces of muscle (wet weight) were taken from a specific site on each muscle, stripped of any tendon strands, weighed analytically (Sartorius, West Germany) and

placed in Corning Pyrex tubes with Teflon caps (American Scientific Products). Macdonald et al. [56] have shown that the distribution of hydroxyproline in normal muscle differs with respect to location within the muscle with the proximal and distal lesions of the muscle containing the highest levels of hydroxyproline. By sampling in the same location on each muscle this variance was eliminated. After the addition of 1.5 ml of six N hydrochloric acid, the tubes were placed in a bacteriological incubator (B Blue Electrical Company, Model No. 100-A, Blue Island, Illinois) where the tissues were hydrolized at 110^{o} C for eighteen hours. After allowing the tubes to cool, Methyl Red indicator (Fisher 0.02 percent solution) was added followed by 3.6 ml of 2.5 normal NaOH added drop wise until the solution turned straw colored. This color change corresponds to a pH of between 6.0 and 7.0. Final adjustments were made with dilute HCL and NaOH. The samples were compared with the standard water tube marked to a volume height of 15 ml and H_2O was added to bring each sample to volume. A stock solution of hydroxyproline (Aldrich S. L/HP440-9) was used to prepare standard dilutions containing from one to five mg hydroxyproline per 2.0 ml volume to be quantitated as controls spectrophotometrically (UV Spectrophotometer Model No. 1332, Beckman Instruments). A linear relationship has been shown between absorbency at 561 nm and hydroxyproline concentration [112]. The experimental samples were diluted

in a similar manner in two volumes to contain hydroxyproline concentration within the standard curve. One ml of Chloramine T Fisher (O-1779 reagent grade) was next added to the samples to initiate hydroxyproline oxidation and allowed to incubate for twenty minutes at room temperature. Next, one ml of perchloric acid (Fisher A-229 reagent grade) was added to each sample in order to destroy the chloramine T. After five minutes, one ml of dimethylamino benzaldehyde (DAB Fisher D71/100) was added and samples were incubated in a hot water bath at 60° C for twenty minutes. The hydroxyproline content of the samples was then quantitated spectrophotometrically against the standard hydroxyproline curve. Hydroxyproline concentration in ug/mg wet weight of rat tibialis anterior muscle was then calculated and values expressed as a percentage of the internal control (nonoperative leg).

F. Histological Studies

Three months after the initial procedure, the nerve grafts of all experimental animals were re-explored, harvested, and fixed in four percent gluteraldehyde cacodilate buffer at pH 7.4 overnight at 4° C. The tissue was then washed with cacodilate buffer with sucrose and post-fixed with one percent osmium tetroxide in cacodilate buffer and dehydrated in graded alcohol. The tissue was then imbedded in epon according to Lufts Formula No. 1. The samples were sectioned with an ultra-microtome

(LKB Huxley, AB Bromma, Sweden) with a glass knife and one-half to one micron thick sections were prepared and examined by light microscopy. Ultra thin sections were also cut with the ultra-microtome stained with uranyl acetate and lead citrate and examined on a Philips P-200 Electron Microscope (Philips Electronic Instruments, Mt. Vernon, New York). Nerve segments harvested from gap control nongrafted segments were processed at the time of removal by the same methods. The animals were sacrificed by carbon dioxide inhalation.

All animals in each group were studied by light microscopy. Two animals in each group were studied by electron microscopy. Sections were taken from the sciatic nerve proximal to the nerve graft, through the proximal, middle and distal graft, and distal to the nerve graft. Sections taken proximal to the graft were used as internal controls. Using the Osteo-Plan (Zeiss, Inc., West Germany) photographic enlargements were made of each nerve segment. These enlargements were used to count the total number of myelinated nerve fibers in each nerve segment. In addition, nerve cross sectional area and perimeter were calculated according to a program for such calculations by the Osteo-Plan.

The gap animals were also re-explored. If any evidence of fibrous or neural bridging were encountered, samples of tissue were harvested, fixed, and prepared in the above manner.

G. Miscellaneous Testing

In animals in which self-mutilation had occurred, the number and location of missing toes was reported. Each animal's weight was recorded at the time of the initial procedure and before sacrificing (Ohaus 2610 Dial-O-Gram Balance). At the time of re-exposure of the nerve graft segment, each site was graded according to scar tissue formation on a scale of one to three indicating: 1) minimal scar tissue approximating normal anatomy, 2) moderate fibrous proliferation, nerve segment clearly visible, and 3) heavy fibrous proliferation, nerve graft obscured by scar tissue formation. Each animal's score was reported in the form of a scar index (see Fig. 13,14).

H. Statistical Analysis of Data

Statistical analysis was performed on all experimental data utilizing the SAS statistical analysis package on the Yale University Computer Center mainframe computer. An analysis of variance was conducted among and between the three experimental groups by means of Kruskal-Wallis ANOVA nonparametric tests. Individual comparisons made between the two grafted groups, between operative and nonoperative groups and within each group between operative and nonoperative legs were tested for probabilities and levels of significance by means of the Dunn analysis for multiple comparisons using rank sums and with the Student's t-test

where appropriate. Linear regression analysis was used to analyze the relationship between hydroxyproline concentration and tibialis anterior weight and values fitted to a general linear model. Estimates for the regression parameters of this model were determined.

IV RESULTS

A. Functional Testing

The results of functional testing support our hypothesis that fresh and cryopreserved autografts are not statistically different in their ability to affect axonal regeneration across the graft segment as evidenced by functional recovery but are statistically superior in this ability to gap control animals (see Table 1-3).

Preoperative and postoperative toe spread (TS) (the linear distance from the center of the print of the first toe to the center of the print of the fifth toe) measurements were made and values expressed as a percentage of preoperative normal values. Each animal's nonoperative leg served as its own control in walking track measurements. Significant differences ($p < 0.05$) existed in each group when pre- and postoperative values were compared (see Fig. 3). Postoperative values for toe spread (a measure of the function of the peroneal nerve branch) for the "experimental" side were expressed as a percentage of the corresponding value measured for the control side. These values were .434 (SD, .011) for the fresh grafted group, and .451 (SD, .011) for the cryopreserved grafted group. The value for the gap control group was .496 (SD, .004). These values do not represent statistically

significant differences in return of function between the three groups.

In a further determination of return of function, each animal's operative foot was measured for range of motion. The normal range of motion for extension at the ankle in the rat is 170°. This value was uniformly attained in range of motion measurements on nonoperative control feet. The mean range of motion in terms of degrees of flexion contracture (170° minus the measured motion) in the fresh grafted group was 18° (range 30° to 5°) and in the cryopreserved grafted group 14° (range 25° 5°). In the graft control group, the flexion contracture was 49° (range 82° to 28°)(see Fig.4). These values represent no statistically significant difference in return of function as measured by flexion contracture in the two autografted groups. There was, however, a difference between the grafted and gap control animals ($p < 0.01$) representing an improvement in functional return in the grafted animals (see Fig. 15,16).

At the time of sacrifice, the rats' tibialis anterior muscles were harvested bilaterally and their wet weights determined. The tibialis anterior is a long, narrow muscle that dorsiflexes the ankle and inverts the foot. It received innervation from the deep peroneal nerve, a distal branch via the common peroneal nerve of the sciatic nerve. In each animal, the nonoperative tibialis anterior muscle served as a control for the weight of the muscle on the

operative side. There was no statistically significant difference between the control tibialis anterior weight value among the three groups (see Fig.5). The data did, however, represent a statistical difference between operative and nonoperative muscle weight within each group ($p < 0.05$)(see Fig.17,18). The mean weight for a tibialis anterior muscle innervated via a fresh nerve graft was .5877 gm (SD, .0746 gm) and innervated by a cryopreserved nerve graft .5801 gm (SD, .0935 gm). Functionally, no significant difference is seen between the two grafted groups with respect to reinnervation of the tibialis anterior muscle as evidenced by the preceding data. Functionally the results are superior when the reinnervation of the tibialis anterior is compared in the grafted groups to the gap controls where the average weight of the tibialis anterior was .2089 (SD, .0844)(see Fig.5). This difference is significant at the $p < 0.01$ level.

B. Sensory Testing

The results of sensory testing in our experimental model were equivocal in their support for our hypothesis that sensory return is equal in the fresh and crypreserved grafted groups and superior to that in the gap control animals. Sensory testing was performed in all rats at two week intervals and at the time of sacrifice as described in the Methods section. At two weeks, without exception, rats had regained some degree of sensibility in the operative

foot. The sensory response to painful stimuli was, however, always decreased in comparison to the intact control foot. Most rats retained this sensibility even after the nerve graft segment had been removed. Few rats regained minimal sensation to the lateral most toes. No rats in the gap control group experienced return of lateral toe sensation of any kind. A comparably large number of rats in the gap control group showed evidence of self mutilation of the lateral toes of the operative foot when compared to grafted animals (see Fig.19).

C. Hydroxyproline Assays

The results of the hydroxyproline assay confirm our hypothesis that fresh and cryopreserved nerve graft segments are not statistically different in their ability to reinnervate distal musculature but are statistically superior in this ability to gap control animals. The whole muscle mean intramuscular hydroxyproline content of normal tibialis anterior muscle as measured from the nonoperative leg of each animal was not statistically different among the three groups (see Fig.6). Assays were run on two sample concentrations from each tibialis anterior muscle, and results were reported as hydroxyproline content in the operative leg divided by hydroxyproline content in the control leg expressed as a percentage. There were no significant differences found in the values obtained by the two separate assays within each group. Results showed no

statistical difference between the hydroxyproline content of tibialis anterior muscle innervated via fresh or frozen sciatic nerve grafts (see Fig.7). The mean hydroxyproline percentage value in the fresh grafts was 1.382 (SD, .305) and 1.527 (SD, .405), and in the cryopreserved grafts 1.655 (SD, .497) and 1.660 (SD, .456). The results are, however, superior when the hydroxyproline level of the tibialis anterior was compared in the grafted groups to the gap controls where the average hydroxyproline percentage values were 4.315 (SD, 1.965) and 4.869 (SD, 2.351)(see Fig.8). This difference is significant at the $p < 0.01$ level.

D. Histological Studies

The results of histological analysis confirm our hypothesis that no difference exists between the ability of fresh and cryopreserved grafts to serve as a conduit for axonal regeneration. Fresh and frozen autografts cannot be distinguished from one another grossly at the light microscopic level with respect to cellular reaction, macrophage response, or connective-tissue proliferation. Evidence of regeneration was observed at three months in all animals receiving a fresh or a frozen graft (see Fig. 9-12). Electron microscopy demonstrated regenerating fiber units and viable Schwann cells in all grafts. Again no evidence of cellular infiltrates around the nerve graft was seen. No evidence of regeneration or bridging was seen in any of the gap control animals.

Control values for axonal counts were obtained from the sciatic nerve segments removed from the gap control animals. Myelinated nerve fiber counts were made and neural cross sectional area measured. The mean fiber density for the proximal segment was 4,221 (SD, 262), and for the distal segment 3,885 (SD, 744). These values do not represent a statistical difference. The calculated proximal to distal mean fiber density ratio in these control nerve segments was .92 (SD, .15)(see Table 3).

In the animals receiving fresh nerve grafts, the mean fiber density calculated as above for the nerve segment proximal to the graft was 4,569 (SD, 453) and for the nerve graft segment, itself, 5,025 (SD, 457). The calculated mean fiber density ratio between the graft and the segment proximal to the graft in the animals receiving a fresh graft was 1.13 (SD, 0.16)(see Table 1).

In the animals receiving cryopreserved nerve grafts, the mean fiber density for the nerve segment proximal to the nerve graft was 4,195 (SD, 728) and for the nerve graft segment 5,126 (SD, 297). The calculated mean fiber density ratio was 1.26 (SD, 0.30)(see Table 2). There is no significant difference between the fresh and cryopreserved grafted animals with respect to the number of regenerated myelinated nerve fibers within the grafted segments. Examination of single fibers regenerating through grafts showed that relatively many more fibers of small diameter are present in the grafted (fresh or cryopreserved)

segments than in the normal nerves (see Fig. 1,2). But again no significant difference could be detected with respect to axonal number or mean fiber density between grafts, either fresh or cryopreserved, and normal nerves.

E. Hydroxyproline Concentration vs. Wet Weight of Tibialis Anterior Muscle

Linear regression analysis was performed to analyze the relationship between hyproxyproline content and wet muscle weight of the tibialis anterior muscle at time of sacrifice. Comparison values were fitted to a general linear model (see graph) and estimates for the regression parameters of this model were determined. Statistical analysis of the model revealed an R^2 value of 0.716 and an F value of 85.58. These values are significant at the $p < .0001$ level (see Fig.20).

V. DISCUSSION

A. Functional Results

As previously discussed, although easily assessed in man, functional recovery is difficult to measure in experimental animals. We attempted to quantify functional return by adapting the method of deMedinaceli et al. [24], devised to evaluate sciatic nerve damage from measurements of the hind feet of walking rats, to our experimental model. We found their basic assumption that all measurements carried equal significance flawed. The most reproducible measurement in our series was the toe spread measurement (TS). Measurements of paw length (PL) varied in response to animal cooperation with the test and proper application of developing fluid. In addition, it was difficult in paw length measurements to define points of reference. In measuring intermediate toe span (IT), most points were combined or difficult to resolve. Gutmann [39] has shown in his classic article in 1942 that loss of toe spread is the most reliable measurement of peroneal nerve injury in rabbits. The functional test described by deMediceli does work for animals whose nerves are close to normal as in the case of a crush injury. This is supported by deMedinaceli's data and from unpublished work in our laboratory (Trumble et al.). When, however, the normal architecture of the nerve is destroyed, the index is less reliable. This is supported by our data and, in fact, by deMediceli's data in his original report in which the

author failed to report follow-up data for the animals with repaired transected nerve injuries.

In the course of functional testing, problems were encountered with self mutilation and flexion contractures involving the operative foot. These problems were converted to statements concerning functional return. The animals in the gap control groups consistently engaged in higher rates of self mutilation and suffered more severe flexion contractures than the grafted animals. Twice as many animals, in fact, suffered self mutilation in the gap control group when compared to fresh or cryopreserved grafted groups. The mean flexion contracture in the gap control group was 50^{o} compared with 20^{o} in grafted animals. No statistical difference was seen in the comparison of the fresh versus the cryopreserved grafts with respect to these parameters.

The wet weight of each animal's tibialis anterior muscle was measured at the time of sacrifice. This was performed as a test of functionally significant reinnervation of distal musculature. Results showed no statistical difference between the weights in the fresh and cryopreserved grafted animals. Each grafted group was, however, statistically superior in terms of results when compared to gap control animals.

A comparison of our results with those of previous investigators with respect to functional recovery is difficult due to the fact that few have undertaken such

determinations. As previously mentioned, many authors [21,56,81,94] have used maximum conduction velocities to draw functional conclusions from their grafting experiments despite the fact that Almquist [4] has proven the lack of correlation between clinical results and nerve conduction velocity. In light of these findings, we have not incorporated nerve conduction velocity measurements into our experimental model.

Hydroxyproline concentrations were determined from the rat tibialis anterior muscles of each animal as a further test of functional return. Intramuscular hydroxyproline levels are known to be a direct indication of collagen content. Macdonald [56] et al. have shown, through reinnervation studies, a correlation between function and collagen content with increasing fibrosis and loss of muscle fiber mass indicative of poorer functional recovery. We have adapted their methods to our model to quantitate functional recovery in terms of hydroxyproline concentration. Ours is the first application of this assay to nerve grafting experiments. Our results show statistically significant differences when expressed as net hydroxyproline concentration or when each value of hydroxyproline content for the experimental leg is expressed as a percentage of the corresponding value measured from the control side between grafted and gap control animals. In addition, differences are seen between grafted and control legs within each group. No

stastistically significant difference, however, exists between the fresh nerve allografted animals and those receiving cryopreserved autografts.

In conclusion, from our data it was not possible to differentiate fresh nerve autografts from cryopreserved autografts in terms of functional return with respect to direct postoperative measurements, muscle mass or hydroxyproline content. Results were, however, superior in these two grafted groups to those found in the nongrafted gap controls.

B. Sensory Testing

Sensory testing is an excellent marker in humans for functional return with its return preceding that of motor function return. Sensory testing in animals such as the rat, however, has proven difficult from a practical standpoint and in the interpretation of results. Most if not all authors have abandoned sensory function as imprecise because of confusing overlapping innervation and because measurements of sensory function are usually indirect [24,39]. We have through the course of this experiment developed what we feel is a reliable, direct, and reproducible test for sensory functional return.

In the course of our sensory testing experiments, we found return of sensation within the first week postoperatively in all animals in the two grafted groups. Even the gap control animals with a 2.0 cm deficit in the

sciatic nerve experienced this early return of sensibility. At the time of sacrifice, animals retained this sensibility even after a 2.0 cm segment of their sciatic nerve, i.e. the graft was removed. It was noted, however, that the sensibility of the lateral aspect of the rat's foot was markedly decreased in the grafted groups and totally absent in all cases in the nongrafted gap controls.

Cajal [17] has shown as a result of his work in 1928 that return of sensate function involves movement of two interacting populations of axons: regenerating fibers from the severed nerve and sprouts emitted by intact fibers in the skin surrounding the denervated zone. Devor [27] through his investigations of these movements behaviorally and electrophysiologically showed that the rat's foot receives its cutaneous innervation along the saphenous nerve (a continuation of the femoral nerve] and the sciatic nerve. Through crush and ligation studies, Devor found return of sensibility within four days in the sciatic distribution due to invasion of branches of the intact saphenous nerve. The sciatic nerve, itself, was able to reinnervate its distribution of skin twenty days after simple injury. When, however, the sciatic nerve was ligated, early return of sensation was seen, but sensation never returned to the lateral two and a half toes. Therefore, it was concluded that this area of the rat's foot received innervation solely from the sciatic nerve. Sensibility testing in this area can be used as a precise

indicator of functional return of regenerated fibers through our nerve graft segments in the sciatic nerve. Although neither Devor nor subsequent authors anticipated its potential application, Devor's work can be used as a reliable test for sensory functional return in the rat with the return of sensibility to the lateral toe indicating return of regenerating axons down the length of the sciatic nerve to its terminal branches. Only general conclusions can be made as a result of these findings in our current study. However, these findings set the stage for further use of sensory return to the lateral toes of the rat as a quantitative test of functional return in the rat model and will be employed in future investigations.

C. Histology

In the course of our study, we experienced early problems with orientation of peripheral nerve graft segment sections secondary to the need to cut segments small enough to allow penetration of the glutaraldehyde fixative. These problems of only a small practical concern were solved with the use of Karnovsky's fixative. This solution has a better penetrative capacity and allowed us to fix long nerve segments which can be easily oriented to make cross sectional cuts.

Our findings were consistent with those of previous investigators including MacKinnon [58,60,61] et al. working with fresh isografts and allografts and Singh [95,96,97]

working with frozen grafts. What we found was a relative increase in the number of myelinated axons of smaller diameter in the nerve graft segments when compared to proximal segments or normal control axons. Fresh and cryopreserved autografts proved equally effective in potentiating axonal regeneration with no significant differences in either total axonal counts or axonal densities found within the two groups.

The rats' "superlative, regenerative capacity" in peripheral nerve injury as described by MacKinnon [128] in a recent published report was not seen in our model. None of the thirteen rats which received a gap of 2.0 cm in their sciatic nerve were able to breech this deficit. In fact, little evidence of axonal sprouting was seen at all. Biopsy sections were taken in all rats which grossly showed any evidence of fibrous spanning and in two rats with no evidence of spanning at all. All sections were prepared and studied microscopically for presence of neural tissue. In these sections, no evidence of neural regeneration was seen at either the light or electron microscopic level. These findings are consistent with those of Lundberg [54], Bucko and Steinmuller [14], and Ducker and Hayes [27] using similar experimental models. With our data we may dispute MacKinnon's findings on histological grounds and reject the notion that gaps of this size can be spontaneously bridged by axonal sprouting without a nerve graft or other conduit to aid such regeneration.

No evidence was seen at the light or electron microscopic level of a cellular reaction or macrophage response in any of the fresh or cryopreserved grafted nerve segments. From these histological findings, we found no evidence that freezing an autograft nerve segment under optimal conditions and with the use of a cryoprotectant agent does in itself alter the grafts' immunogenicity.

In conclusion, from our data it was not possible to differentiate freshly grafted autograft segments from cryopreserved autografts by connective tissue proliferation, cellular reaction, macrophage response, distal myelination, axonal counts, axonal densities, or loss of Schwann cells noted on electron microscopy.

VI. Conclusion

Our results have shown through functional tests, sensory tests, histological analysis, axonal counts and hydroxyproline assays that fresh and cryopreserved autografts are equal in their ability to potentiate regeneration of axons and return of function in the rat. As previously mentioned, these findings have immediate clinical applicability in cases of segmental nerve loss in a wound not immediately ready for grafting or in a patient not yet stabilized for major surgery. If perfected, cryopreservation affords the surgeon the ability to perform reconstructive surgery in an elective rather than an emergent setting. With a method for cryopreserving peripheral nerve tissue in place, we can now look at isograft and allograft models. The next step is to explore the role of immunosuppression in frozen nerve allografts with the ultimate goal of developing practical techniques for vascularized peripheral nerve allografts.

FIGURES, TABLES, and ILLUSTRATIONS

FIGURE 1.

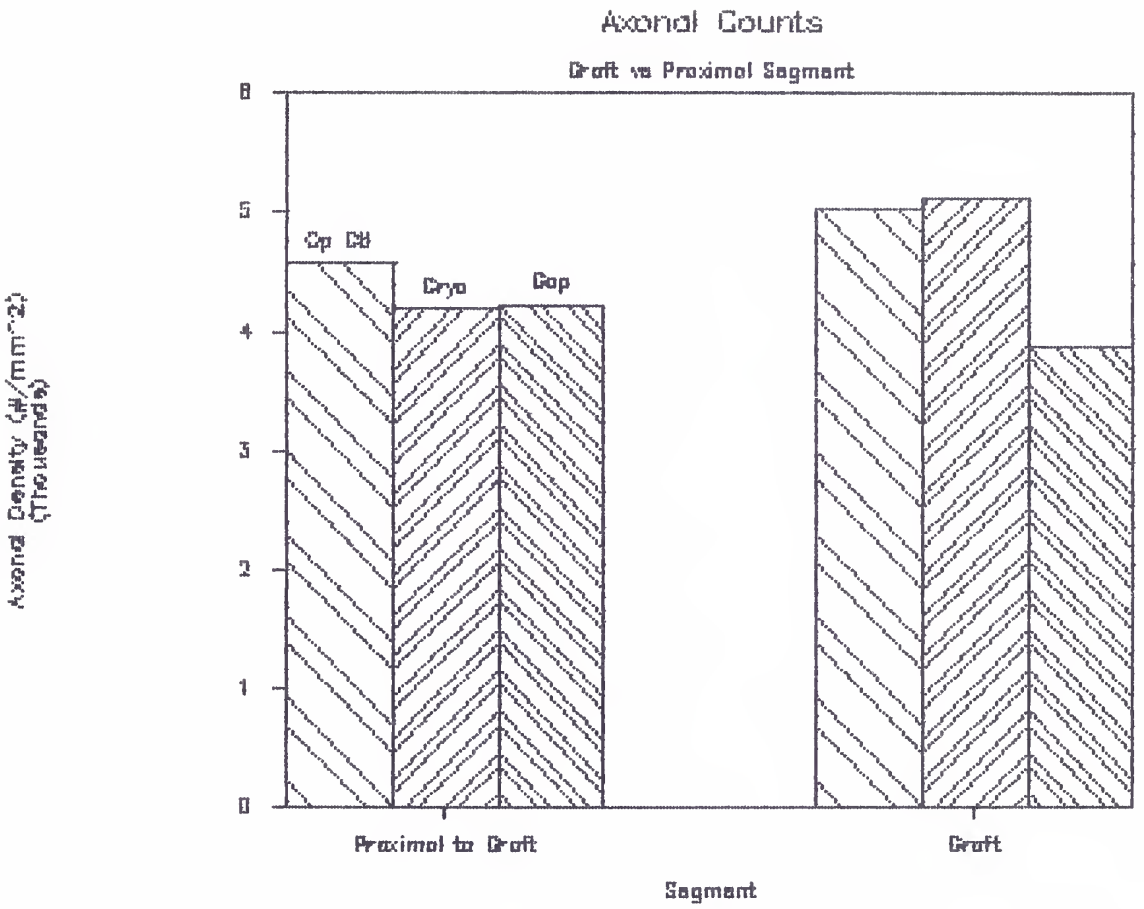


Fig. 1. Axonal densities in the proximal to graft and graft portions of the sciatic nerve. Note the increased density of regenerating fibers in the graft segments. The Gap bars represent normal nerve segments taken from Gap Control animals at the time of initial procedure.

FIGURE 2.

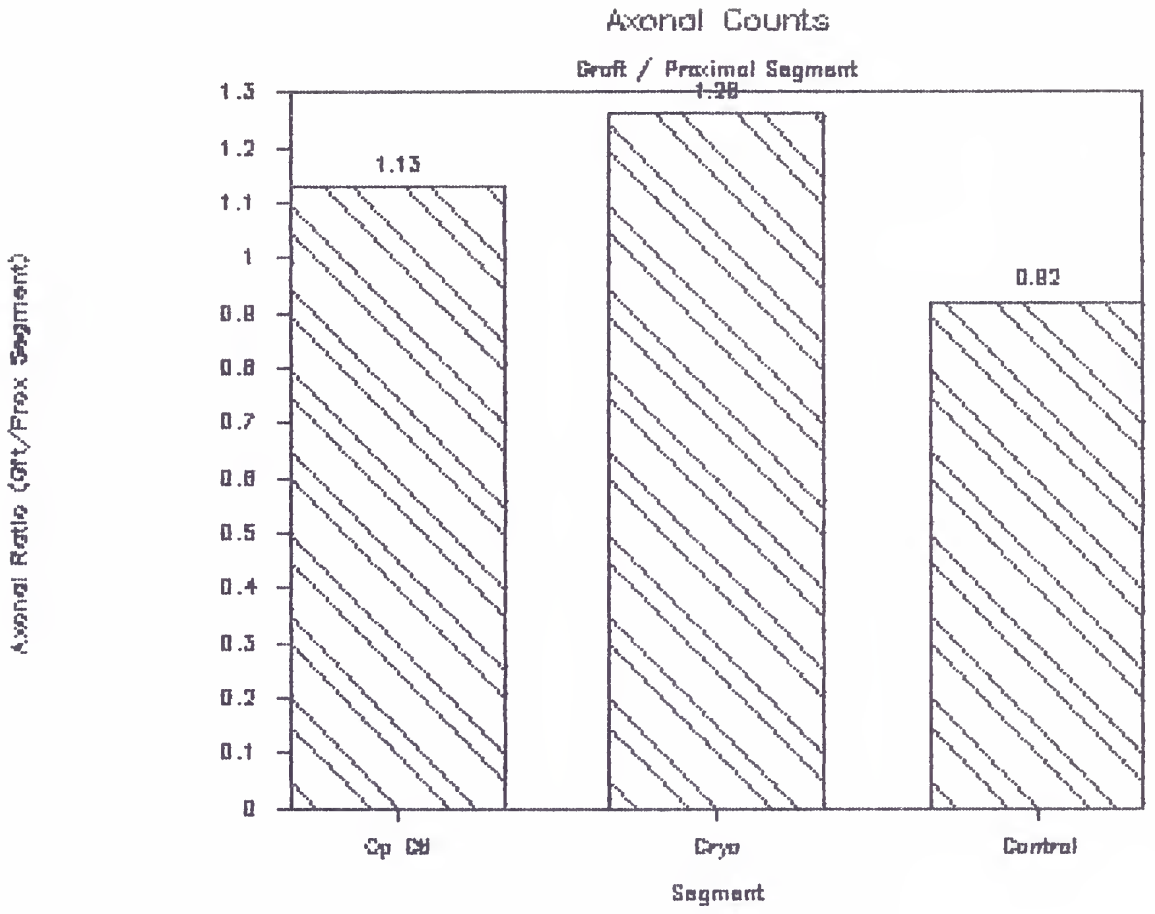


Fig. 2. Axonal densities expressed as graft segment values as a percentage of proximal to graft control segment values in the sciatic nerve. Note the increased axonal density in the grafted animals when compared to control values.

FIGURE 3.

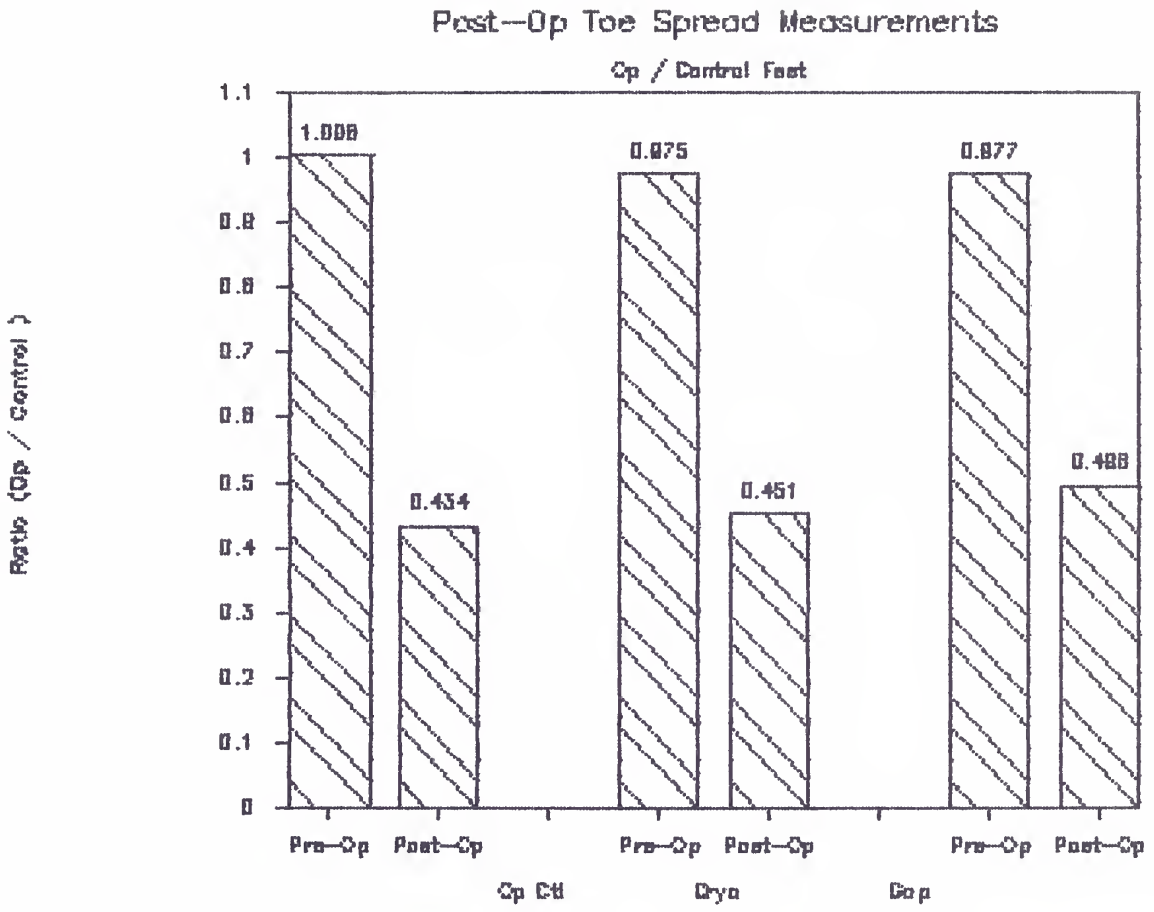


Fig. 3. Preoperative and postoperative toe spread measurements (TS) expressed as a percentage of the control value obtained in the non-operative foot. No statistically significant difference is seen among the three groups.

FIGURE 4.

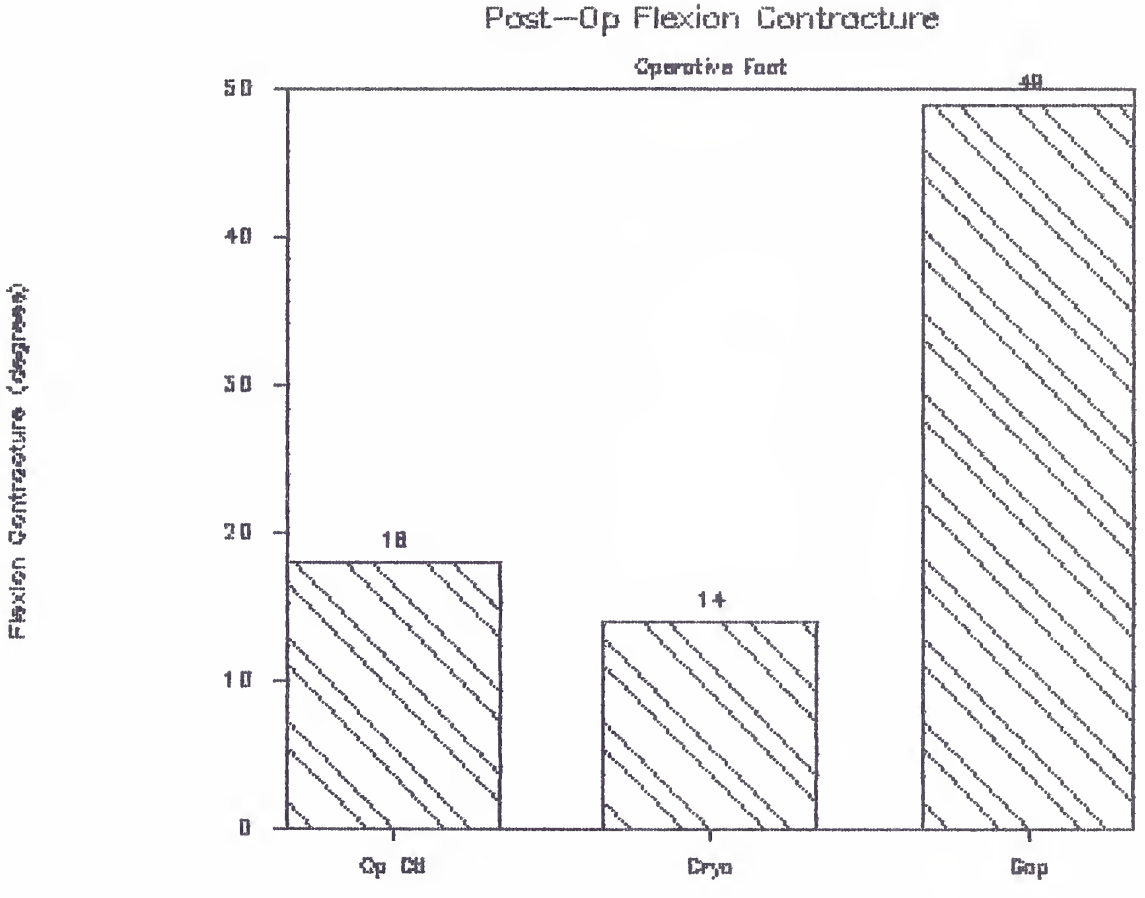


Fig. 4. Postoperative flexion contracture measured at the time of sacrifice in operative feet. The less severe contractures in the grafted groups represent an improvement in functional return in the grafted animals.

FIGURE 5.

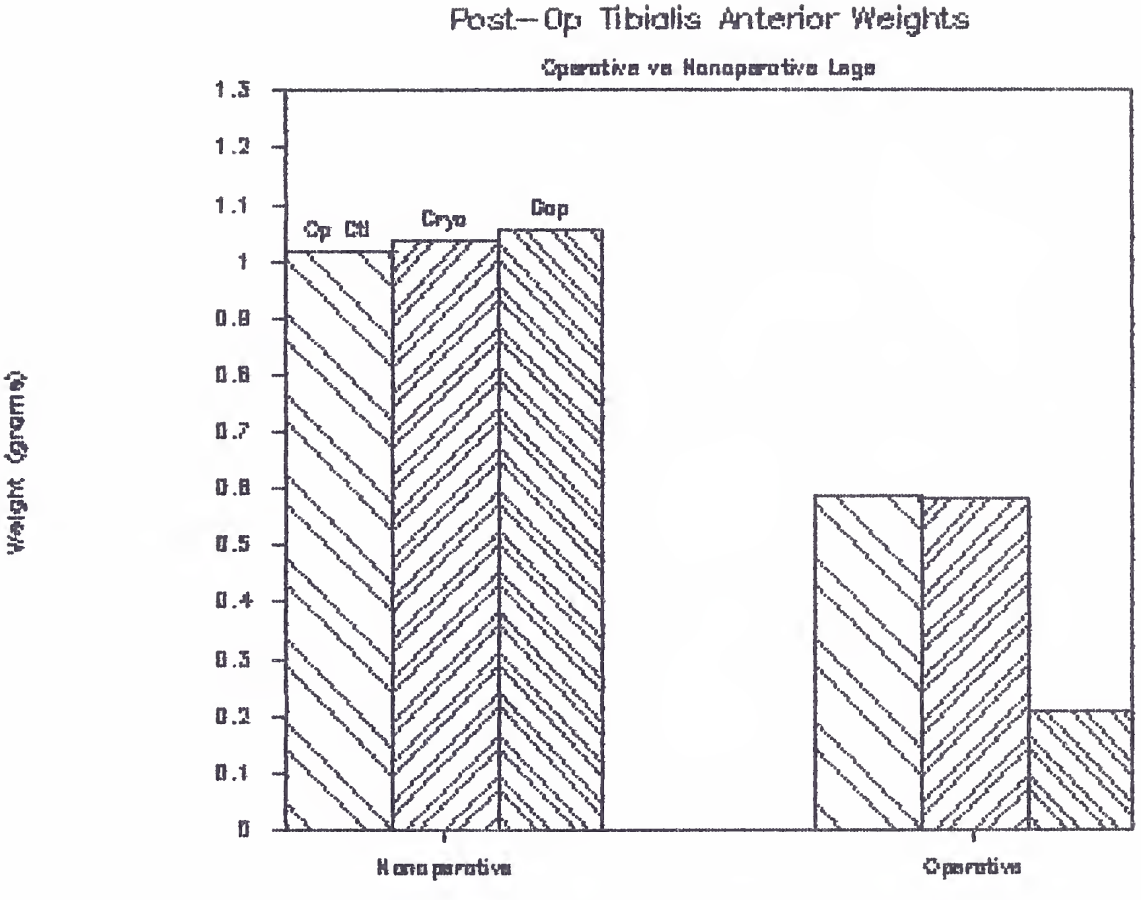


Fig. 5. Postoperative Tibialis Anterior (TA) weights in the operative and nonoperative legs. A significant difference is seen when the reinnervation of the TA is compared in the grafted groups to the nongrafted Gap Controls as evidenced by muscle weights. Again no difference is seen between fresh and frozen nerve grafts.

FIGURE 6.

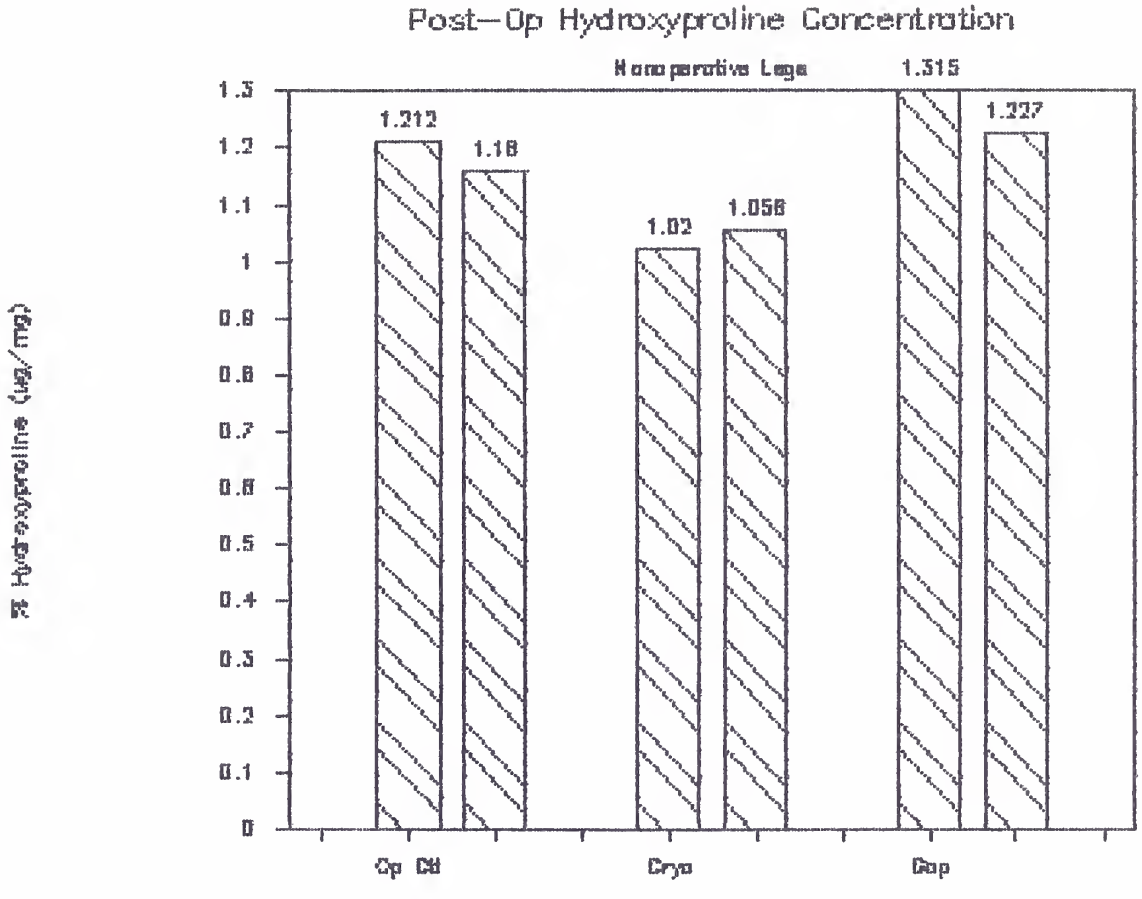


Fig. 6. Postoperative whole muscle mean intramuscular Hydroxyproline (HP) content of normal TA muscle as measured from the nonoperative leg. No statistical difference is seen among the three groups.

FIGURE 7.

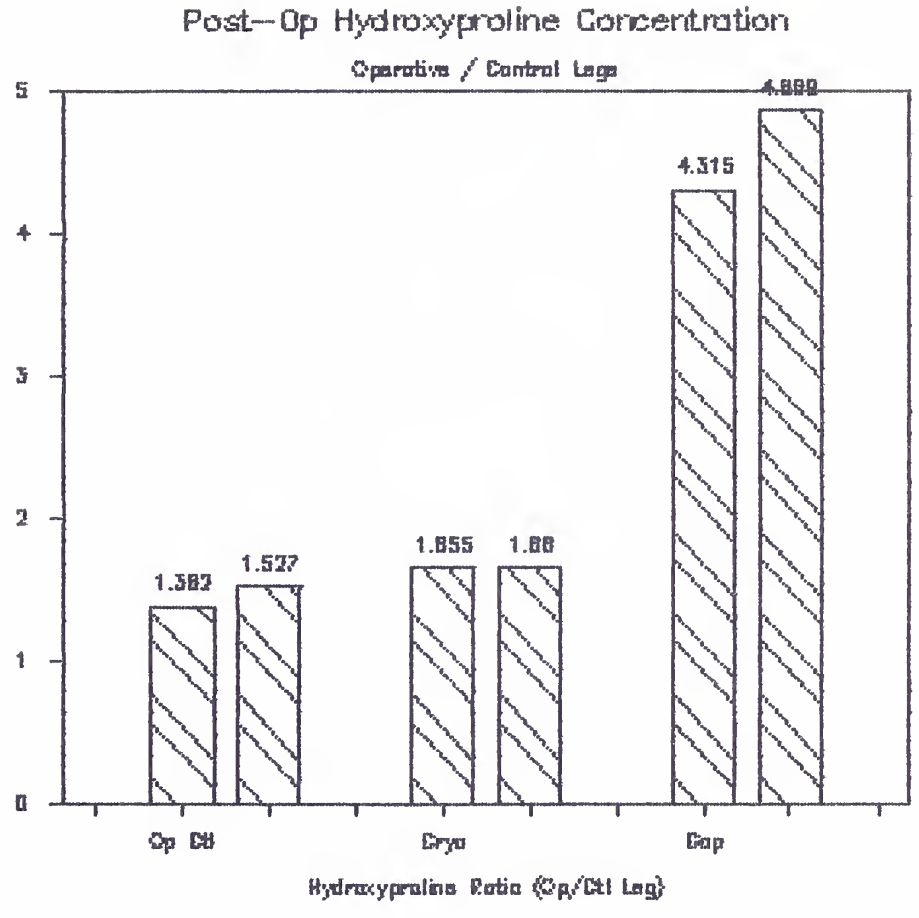


Fig. 7. Postoperative whole muscle mean intramuscular Hydroxyproline (HP) content of reinervated TA muscle as measured from the operative leg. Note the marked increase in HP concentration in the nongrafted Gap Animals. No statistical difference is seen among the grafted groups.

FIGURE 8.

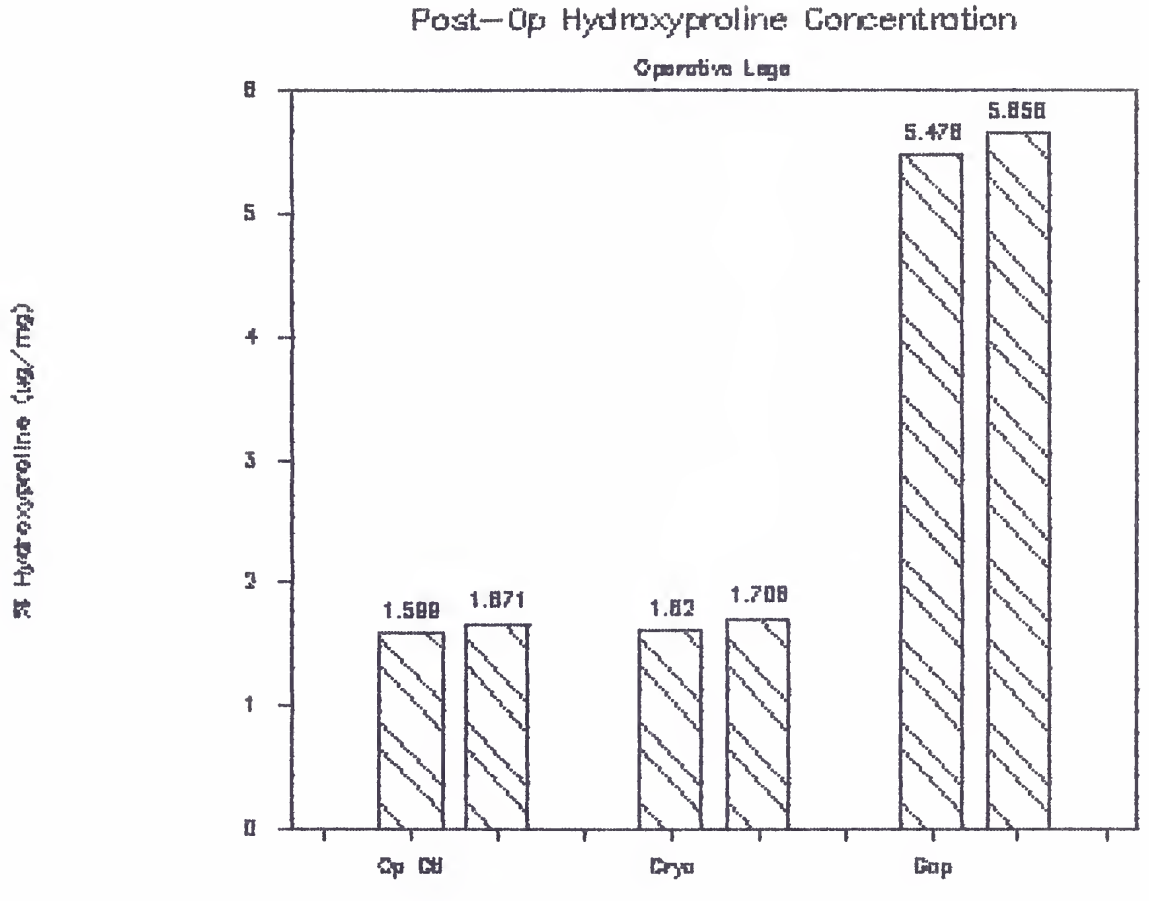


Fig. 8. Postoperative HP concentration expressed as a percentage of control values obtained from the nonoperative leg. Results show no difference in the fresh or frozen grafted groups. Results in grafted groups are superior to those in Gap Control animals.

TABLE 1.

Data From Fresh Grafted Group

Rat Leg	Group	Grafting Weight (gms)	End Weight (gms)	T.A. Weight (gms)	% HP 1 (ug/mg)	% HP 2 (ug/mg)	% HP 1 OP/Ctl	% HP 2 OP/Ctl	Function Test Pre-Op PL	TS	IT	A-Op Ts Op/Ctl
1L	Ctl	372.7	511.7	1.0678	1.214	1.169	1.480	1.392	1.65	1.85	1.10	1.027
1R	Op Ctl			0.6142	1.797	1.627			1.65	1.90	1.15	
2L	Ctl	359.5	506.9	1.0719	1.613	1.518	1.023	1.030	1.85	1.70	1.00	1.000
2R	Op Ctl			0.6429	1.650	1.563			1.65	1.70	0.90	
3R	Ctl	344.6	473.7	0.9811	1.373	1.345	1.223	1.312	1.10	1.80	1.00	0.944
3L	Op Ctl			0.5274	1.679	1.765			1.10	1.70	1.10	
4R	Ctl	311.5	478.5	0.9893	1.321	1.208	1.134	1.300	1.50	1.80	1.00	0.944
4L	Op Ctl			0.5298	1.499	1.590			1.50	1.70	1.10	
5L	Ctl	333.5	483.5	0.9965	1.768	1.742	1.094	1.197	1.50	1.80	1.10	1.056
5R	Op Ctl			0.6196	1.934	2.086			1.60	1.90	1.10	
6L	Ctl	318.8	435.6	0.9176	1.013	0.985	1.819	2.010	1.80	1.80	1.20	0.944
6R	Op Ctl			0.5944	1.843	1.980			1.60	1.70	1.10	
7L	Ctl	320.0	570.3	1.1087	1.186	1.102	1.221	1.386	1.65	1.55	0.90	1.065
7R	Op Ctl			0.7412	1.449	1.527			1.65	1.65	1.10	
8L	Ctl	317.6	450.0	0.8872	1.214	1.206	1.322	1.363	1.70	1.85	1.10	1.027
8R	Op Ctl			0.5147	1.606	1.644			1.75	1.90	1.10	
9R	Ctl	334.5	587.1	1.1299	0.601	0.550	2.004	2.441	1.70	1.70	1.00	1.000
9L	Op Ctl			0.4855	1.205	1.342			1.60	1.70	0.95	
10L	Ctl	313.7	500.7	1.0391	1.034	0.950	1.377	1.705	1.65	1.70	0.85	1.029
10R	Op Ctl			0.5514	1.423	1.621			1.65	1.75	0.90	
11R	Ctl	314.0	500.9	1.0105	1.000	0.981	1.500	1.665	1.60	1.55	0.85	1.032
11L	Op Ctl			0.6440	1.500	1.634			1.60	1.60	1.00	
MEAN		330.9	499.9 CTL	1.0181	1.212	1.160	1.382	1.527				1.006
			OP	0.5877	1.599	1.671						
STD DEVIATION				0.0751	0.316	0.314	0.305	0.405				0.002
				0.0746	0.212	0.207						
VARIANCE				0.0056	0.100	0.099	0.093	0.164				0.000
				0.0056	0.045	0.043						
STD ERROR OF MEAN				0.0227	0.095	0.095	0.092	0.122				0.001
				0.0225	0.064	0.063						

LEGEND

TA = Tibialis Anterior
HP = Hydroxyproline
PL = Paw Length (mm)
TS = Toe Spread (mm)
IT = Intermediary Toe Spread (mm)
A-Op TS = Pre-Op Toe Spread

TABLE 1. (cont.)

Function Test Post-Op PL	TS	IT	P-Op TS Op/Ctl	Toes Missing	Flex Contr (dgs)	PG Ax Ct	PG Area (mm^2)	Graft Ax Cts	Graft Area (mm^2)	PG Axons mm^2	Gft Axons mm^2	Gft/PG Axon #
1.65	1.85	1.20	0.459									
1.60	0.85	0.80		intact		7894	1.5868	3,816	0.9034	4,974.79	4,224.22	0.85
2.10	2.10	1.20	0.190									
2.20	0.40			# 4,5	140	8934	2.0205	7,265	1.4156	4,421.68	5,132.24	1.16
2.00	2.00	0.90	0.549									
2.90	1.10			intact	150			3,494	0.8046		4,342.36	
1.75	1.65	1.25	0.424									
2.30	0.70	0.60		intact	140	8881	1.9560	3,735	0.7334	4,540.29	5,092.66	1.12
1.90	1.90	1.00	0.526									
2.90	1.00			intact		8577	1.8104	5,037	0.9244	4,737.63	5,448.80	1.15
2.30	2.10	1.10	0.429									
2.10	0.90	0.90		intact		6628	1.6298	4,743	0.8643	4,066.74	5,487.68	1.35
2.00	2.10	1.10	0.524									
2.50	1.10			# 3,4,5	165	6730	1.8941	4,301	0.8786	3,553.21	4,895.34	1.38
2.00	2.00	1.10	0.450									
2.90	0.90			intact	165	9526	1.9149	4,289	0.7872	4,974.76	5,448.47	1.10
2.30	1.90	1.10	0.420									
2.80	0.80			intact	145	8620	1.7664	4,496	0.8313	4,879.98	5,408.51	1.11
2.40	1.90	1.00	0.369									
3.00	0.70			intact	155	10644	2.2141	7,311	1.5963	4,807.46	4,580.06	0.95
2.00	1.75	1.10										
3.10				# 3,4,5	150	8596	1.8143	7,791	1.4949	4,737.80	5,211.57	1.10
			0.434		151	7,730	1.8607	5,116	1.0213	4,569.44	5,024.72	1.13

P-Op TS = Postoperative Toe Spread
PG = Proximal to Graft
Gft = Graft
Ax Ct = Axonal Counts

TABLE 2.

Data From Cryopreserved Grafted Group

Rat Leg	Group	Grafting Weight (gms)	End Weight (gms)	T.A. Weight (gms)	% HP 1 (ug/mg)	% HP 2 (ug/mg)	% HP 1 OP/Ctl	% HP 2 OP/Ctl	Function Test Pre-Op PL	TS	IT	A-Op Ts Op/Ctl
1R	Cry	298.3	383.5	0.7833	1.391	1.334	1.000	0.998	1.65	1.60	1.15	1.094
1L	Op Cry			0.4270	1.391	1.331			1.50	1.75	1.00	
2R	Cry	293.9	532.7	1.1473	1.037	1.093	1.828	1.735	1.70	1.70	1.00	1.000
2L	Op Cry			0.6491	1.896	1.896			1.70	1.70	0.90	
3L	Cry	292.1	494.2	1.0568	0.844	0.846	1.885	1.979	1.60	1.80	1.00	0.944
3R	Op Cry			0.5804	1.591	1.675			1.60	1.70	1.10	
4R	Cry	308.3	520.5	1.0123	1.185	1.220	1.289	1.262	1.60	1.90	1.00	0.947
4L	Op Cry			0.7047	1.527	1.540			1.60	1.80	0.95	
5L	Cry	316.5	505.9	1.2187	0.916	0.865	1.418	1.693	1.60	1.70	1.10	1.000
5R	Op Cry			0.5829	1.299	1.464			1.60	1.70	1.10	
6R	Cry	310.5	514.9	1.1434	0.769	0.824	2.360	2.533	1.60	1.85	1.00	1.027
6L	Op Cry			0.6374	1.815	2.087			1.50	1.90	0.90	
7L	Cry	343.8	598.2	1.2901	0.984	0.891	1.255	1.481	1.55	1.90	1.05	1.000
7R	Op Cry			0.4222	1.234	1.391			1.55	1.90	1.00	
8L	Cry	321.4	535.7	1.0366	0.902	0.942	1.131	1.118	1.65	2.00	1.10	0.900
8R	Op Cry			0.6092	1.020	1.119			1.70	1.80	1.00	
9L	Cry	350.6	517.1	1.0536	0.728	0.856	2.494	2.155	1.70	2.00	0.95	0.950
9R	Op Cry			0.7164	1.815	1.845			1.60	1.90	1.05	
10L	Cry	317.5	470.0	0.9330	1.426	1.503	1.293	1.263	1.55	1.80	0.95	0.861
10R	Op Cry			0.5205	1.845	1.898			1.60	1.55	1.00	
11L	Cry	327.1	492.1	0.8226	1.096	1.181	1.765	1.751	1.60	1.90	1.25	0.974
11R	Op Cry			0.5508	1.935	2.068			1.65	1.85	1.00	
12R	Cry	350.0	501.4	0.9417	0.966	1.114	2.140	1.956	1.60	1.90	0.90	1.000
12L	Op Cry			0.5610	2.068	2.179			1.60	1.90	1.00	
MEAN		319.2	505.5 CTL	1.0366	1.020	1.056	1.655	1.660				0.975
			OP	0.5801	1.620	1.708						
STD DEVIATION				0.1514	0.222	0.221	0.497	0.456				0.004
				0.0935	0.327	0.339						
VARIANCE				0.0229	0.049	0.049						0.000
				0.0087	0.107	0.115						
STD ERROR OF MEAN				0.0437	0.064	0.064	0.143	0.132				0.001
				0.0270	0.094	0.098						

LEGEND

TA = Tibialis Anterior
HP = Hydroxyproline
PL = Paw Length (mm)
TS = Toe Spread (mm)
IT = Intermediary Toe Spread (mm)
A-Op TS = Pre-Op Toe Spread

TABLE 2. (cont.)

Function Test Post-Op PL	TS	IT	P-Op TS Op/Ctl	Toes Missing	Flex Contr (dgs)	PG Ax Ct	PG Area (mm^2)	Graft Ax Cts	Graft Area (mm^2)	PG Axons mm^2	Gft Axons mm^2	Gft/PG Axon #
2.00	1.90	1.00	0.474									
3.00	0.90	0.90		intact	160	6456	1.7970	5,042	0.9188	3,592.65	5,487.59	1.53
2.20	2.10	1.00	0.333									
3.50	0.70			# 3,4,5	165	11254	2.7147	5,276	1.1230	4,145.56	4,698.24	1.13
2.10	2.10	1.20	0.429									
2.80	0.90			intact	160	9088	1.7308	8,781	1.7110	5,250.82	5,132.23	0.98
2.10	2.10	1.20	0.333									
2.80	0.70			# 4,5	165	6703	1.5337	4,264	0.8925	4,370.62	4,777.46	1.09
2.20	2.00	1.20	0.450									
3.00	0.90			intact	160	6896	1.5879	5,368	1.1053	4,342.90	4,856.50	1.12
2.00	2.10	0.90	0.429									
3.50	0.90			intact	165	2985	0.6462	3,898	0.7155	4,619.31	5,447.89	1.18
1.90	1.90	1.10	0.526									
2.90	1.00			intact	145	9384	2.2009	5,340	1.0246	4,263.79	5,211.71	1.22
2.20	2.00	1.00	0.650									
2.80	1.30			intact	160	9466	2.2360	3,931	0.7601	4,233.44	5,171.69	1.22
2.00	2.00	1.00	0.550									
2.70	1.10			# 4,5	160	6760	1.3273	5,837	1.0868	5,092.97	5,370.62	1.05
1.90	2.10	1.10	0.286									
2.50	0.60			#1,2,4,5	145	6106	1.3686	6,000	1.1873	4,461.37	5,053.35	1.13
				intact	160	8020	3.0779	4,831	1.0112	2,605.69	4,777.39	1.83
1.90	2.00	1.10	0.500									
2.90	1.00			intact	145	6152	1.8332	5,957	1.0777	3,355.88	5,527.51	1.65
			0.451		157.5	7,439	1.8378	5,377	1.0512	4,194.58	5,126.01	1.26

P-Op TS = Postoperative Toe Spread
PG = Proximal to Graft
Gft = Graft
Ax Ct = Axonal Counts

TABLE 3.

Data From Nongrafted Gap Control Group

Rat Leg	Group	Grafting Weight (gms)	End Weight (gms)		T.A. Weight (gms)	% HP 1 (ug/mg)	% HP 2 (ug/mg)	% HP 1 OP/Ctl	% HP 2 OP/Ctl	Function Test Pre-Op PL	TS	IT	
1R	Gap	318.2	521.8		1.1260	0.860	0.829	2.229	2.375	1.60	1.55	0.90	1.065
1L	Op Gap				0.4328	1.916	1.832			1.50	1.65	0.95	
2L	Gap	352.2	469.8		0.9424	0.948	0.808	7.888	9.462	1.85	1.90	0.80	0.842
2R	Op Gap				0.1359	7.475	7.648			1.50	1.60	0.85	
3R	Gap	377.1	512.6		1.1617	1.298	1.227	2.030	2.358	1.60	1.70	1.15	1.059
3L	Op Gap				0.2409	2.635	2.894			1.50	1.80	1.05	
4L	Gap	412.4	539.8		1.1113	1.678	1.613	1.448	1.585	1.60	1.95	1.30	0.923
4R	Op Gap				0.3070	2.430	2.556			1.50	1.80	1.20	
5R	Gap	384.2	470.0		1.0318	1.259	1.151	3.947	4.423	1.55	2.00	1.10	0.925
5L	Op Gap				0.1538	4.967	5.090			1.55	1.85	1.10	
6L	Gap	404.5	526.0		1.1553	1.679	1.549	2.862	3.101	1.40	1.65	1.00	1.000
6R	Op Gap				0.2070	4.804	4.804			1.40	1.65	1.05	
7R	Gap	336.9	525.8		1.0810	1.080	0.979	6.568	8.061	1.45	1.75	0.90	1.029
7L	Op Gap				0.1181	7.091	7.888			1.30	1.80	1.10	
8L	Gap	327.4	470.5		0.9344	1.333	1.172	5.993	6.843	1.40	1.75	1.10	0.971
8R	Op Gap				0.1872	7.991	8.018			1.45	1.70	0.95	
9R	Gap	317.4	503.9		1.0753	1.215	1.047	3.934	4.686	1.60	1.80	1.10	1.056
9L	Op Gap				0.1610	4.780	4.905			1.60	1.90	1.15	
10L	Gap	323.0	416.9		0.8752	1.145	1.053	4.931	5.503	1.45	1.85	1.15	0.973
10R	Op Gap				0.2032	5.645	5.795			1.30	1.80	0.90	
11L	Gap	324.1	531.5		1.0478	1.476	1.427	4.605	4.781	1.40	1.80	0.95	0.861
11R	Op Gap				0.2241	6.795	6.821			1.30	1.55	0.95	
12L	Gap	313.0	548.8		1.1411	1.695	1.676	3.329	3.651	1.45	1.75	0.95	0.971
12R	Op Gap				0.2072	5.644	6.119			1.55	1.70	1.10	
13L	Gap	319.0	475.3		1.0249	1.429	1.416	6.328	6.471	1.60	1.70	0.90	1.029
13R	Op Gap				0.1372	9.040	9.161			1.55	1.75	0.95	
MEAN		346.9	501.0	CTL	1.0545	1.315	1.227	4.315	4.869				0.977
				OP	0.2089	5.478	5.656						
STD DEVIATION					0.0909	0.272	0.288	1.965	2.351				0.005
					0.0844	2.211	2.271						
VARIANCE					0.0083	0.074	0.083						0.000
					0.0071	4.887	5.156						
STD ERROR OF MEAN					0.0252	0.075	0.080	0.545	0.652				0.001
					0.0234	0.613	0.630						

LEGEND

TA = Tibialis Anterior
HP = Hydroxyproline
PL = Paw Length (mm)
TS = Toe Spread (mm)
IT = Intermediary Toe Spread (mm)
A-Op TS = Pre-Op Toe Spread

TABLE 3. (cont.)

Function Test Post-Op PL	TS	IT	P-Op TS Op/Ctl	Toes Missing	Flex Contr (dgs)	Prox Ax Ct	Prox Area (mm^2)	Distal Ax Cts	Distal Area (mm^2)	Prox Ax mm^2	Dist Ax mm^2	P/D Axon #
2.20	1.80	1.10	0.500									
3.10	0.90			# 3,4,5	130	7113	1.6530	6,463	1.5892	4,303.18	4,066.79	0.95
2.10	1.90	1.00	0.579									
3.00	1.10			intact	135							
2.10	2.10	1.10	0.476									
3.30	1.00			intact	130							
2.10	2.00	1.10	0.450									
3.10	0.90			intact	140	6643	1.6978	5,620	1.5643	3,912.76	3,592.60	0.92
2.10	2.10	1.10	0.429									
3.10	0.90			intact	120	7645	1.7603	4,871	1.1421	4,343.09	4,265.06	0.98
1.90	2.10	1.20	0.476									
3.10	1.00			intact	125	5370	1.2834	1,574	0.7247	4,184.20	2,172.02	0.52
2.00	2.00	1.20	0.400									
3.00	0.80			# 4,5	90	7972	1.6418	7,583	1.5238	4,855.59	4,976.36	1.02
2.20	1.80	1.00	0.500									
3.50	0.90			# 4,5	110	6047	1.4863	7,982	1.8095	4,068.50	4,411.25	1.08
2.00	1.80	1.00	0.444									
2.30	0.80			# 4,5	88	6649	1.6512	6,679	1.7981	4,026.75	3,714.47	0.92
1.90	2.00	1.20	0.500									
3.30	1.00			intact	130	7137	1.6434	5,285	1.2282	4,342.81	4,303.08	0.99
1.90	1.90	1.10	0.526									
2.90	1.00			# 3,4,5	125	5917	1.4272	5,615	1.4815	4,145.76	3,790.09	0.91
2.40	1.80	1.10	0.555									
3.30	1.00			intact	142	5413	1.3712	5,221	1.4692	3,947.71	3,553.56	0.90
1.80	1.80	0.90	0.611									
3.20	1.10			# 4,5	110	5103	1.1856			4,304.22		
			0.496		121.2	6,455	1.5274	5,689	1.4331	4,221.32	3,884.53	0.92

P-Op TS = Postoperative Toe Spread
PG = Proximal to Graft
Gft = Graft
Ax Ct = Axonal Counts

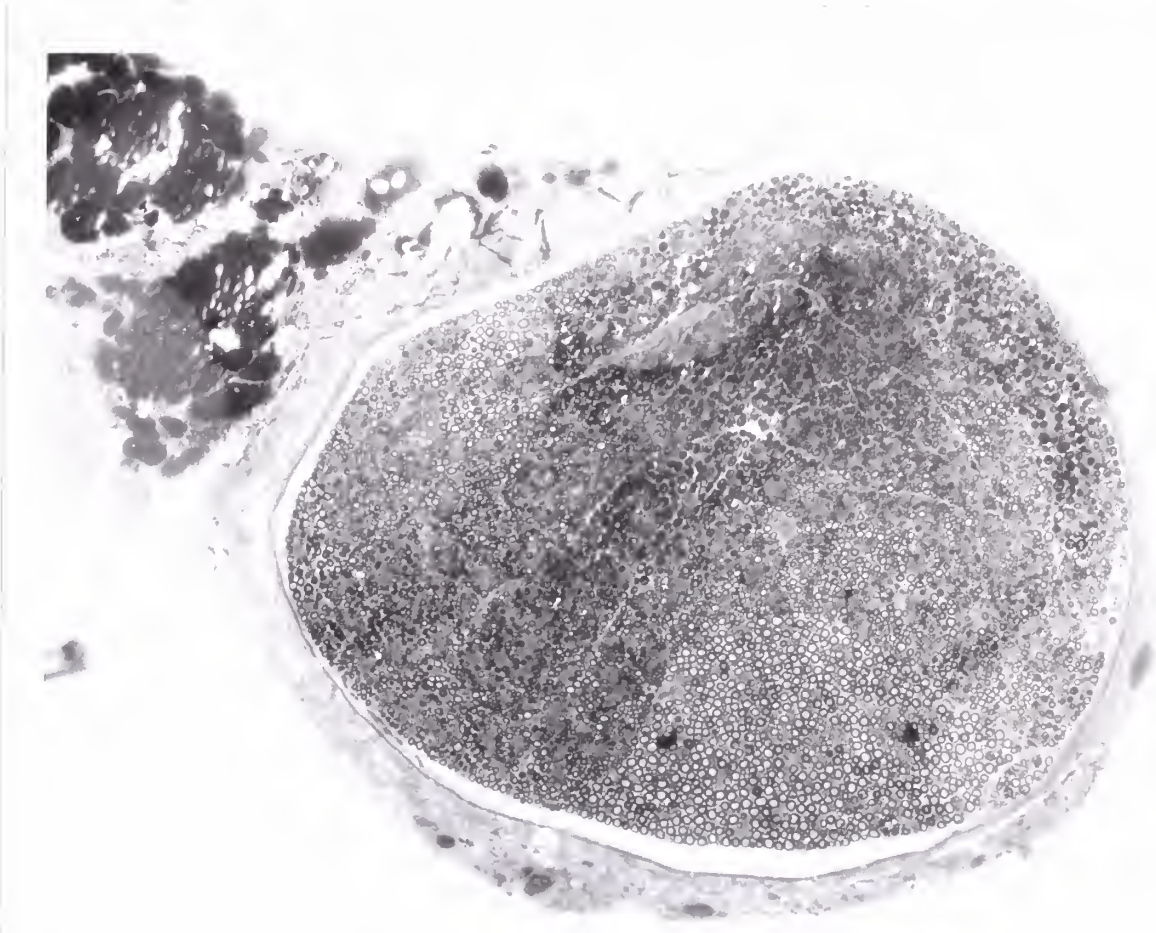


FIGURE 9. Cross sectional photomicrograph of the proximal to graft region of the sciatic nerve of a cryopreserved nerve grafted rat taken three months after grafting.

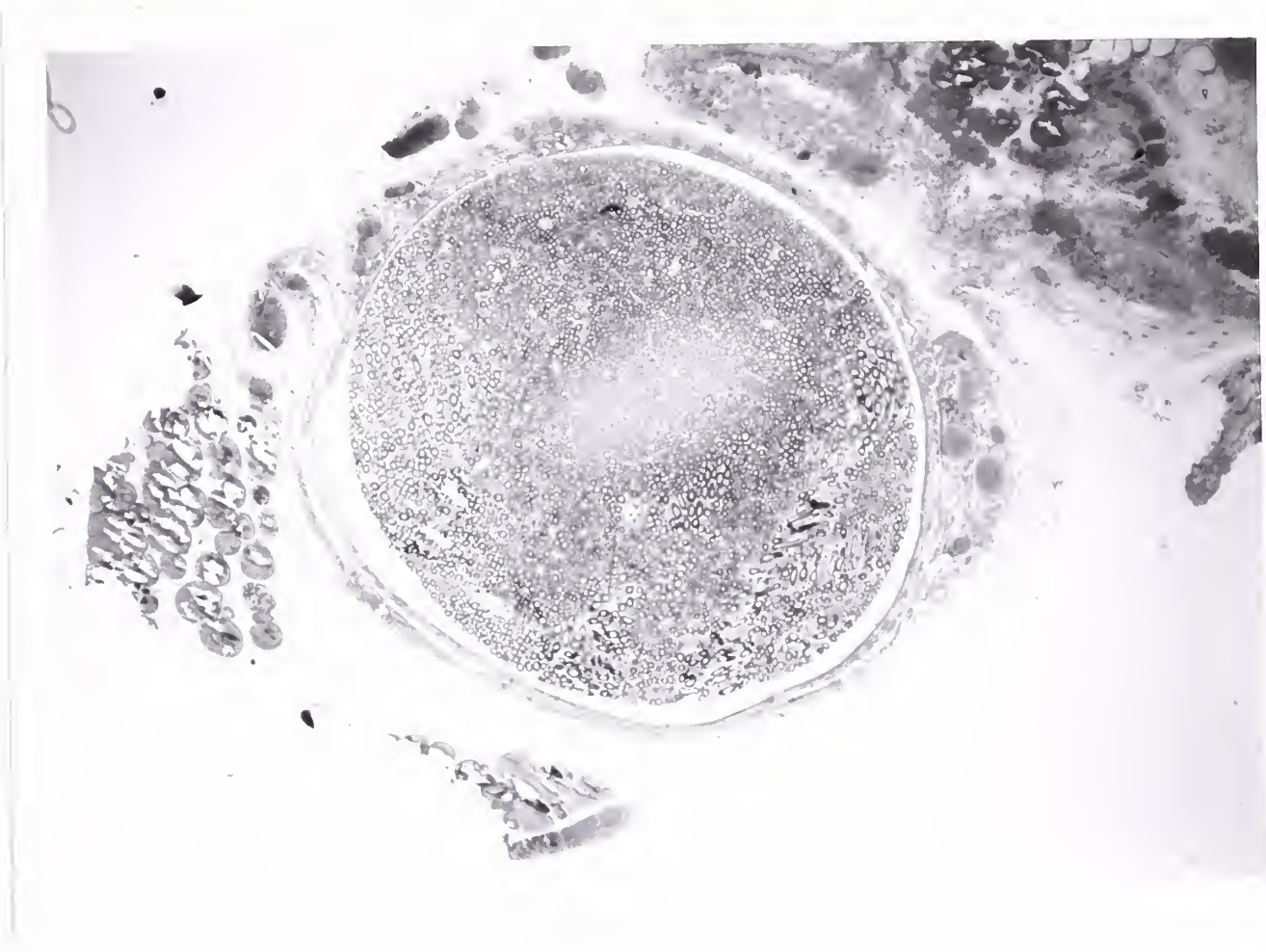


FIGURE 10. Cross sectional photomicrograph of a normal sciatic nerve taken from a gap control animal (#10) in the region where sections were obtained in grafted sciatic nerve segments.

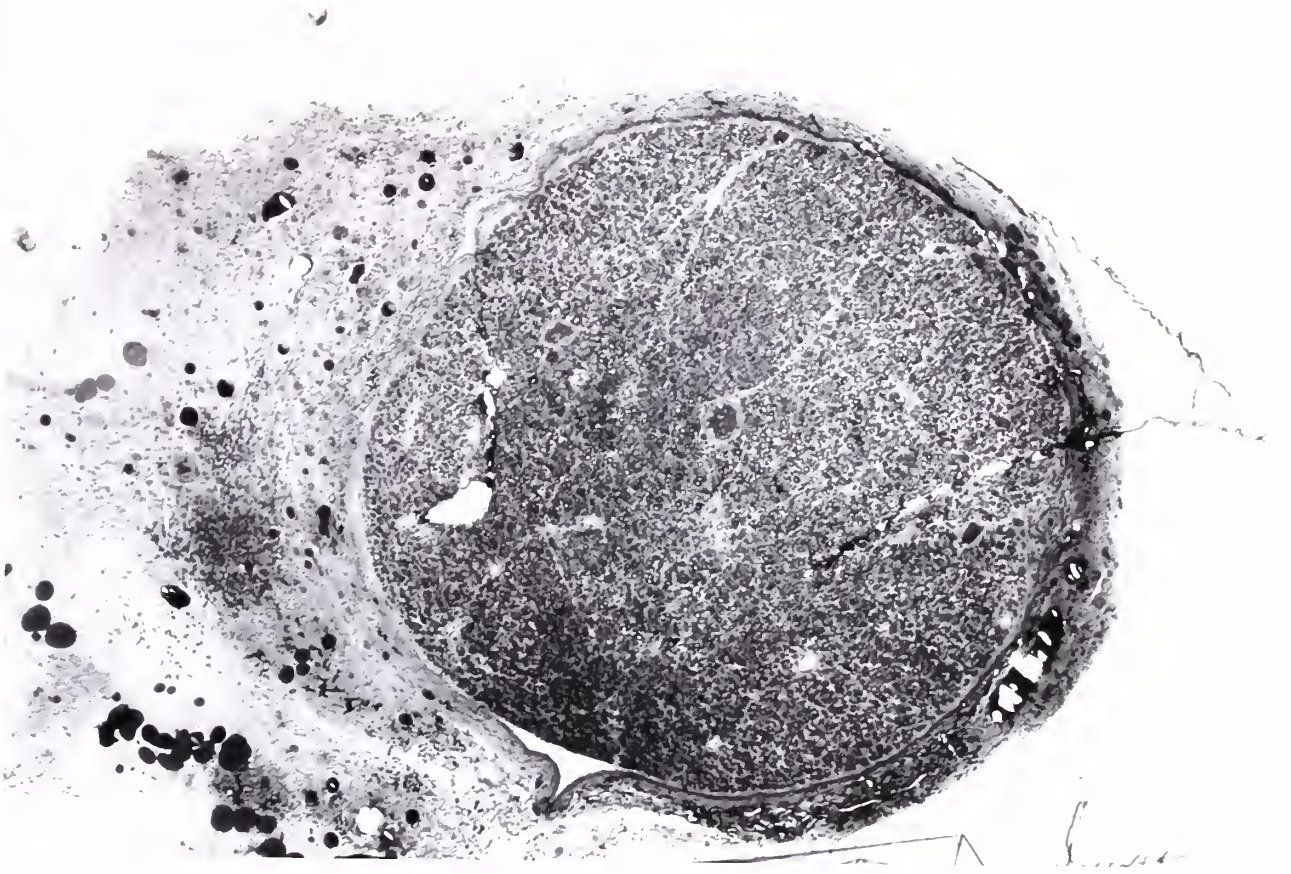


FIGURE 11. Cross sectional photomicrograph of a fresh grafted sciatic nerve taken three months after grafting in the same region as the control pictured in Figure 10. A population of well myelinated nerve fibers is noted.

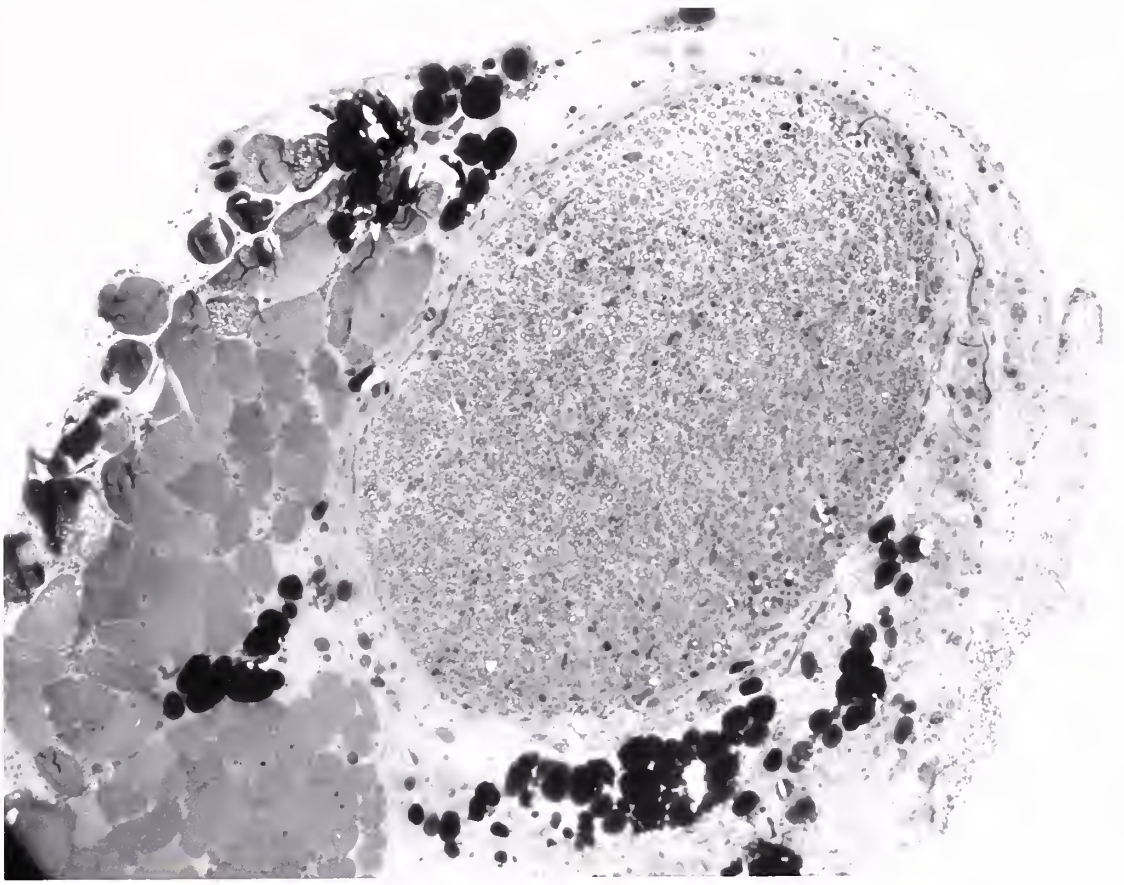
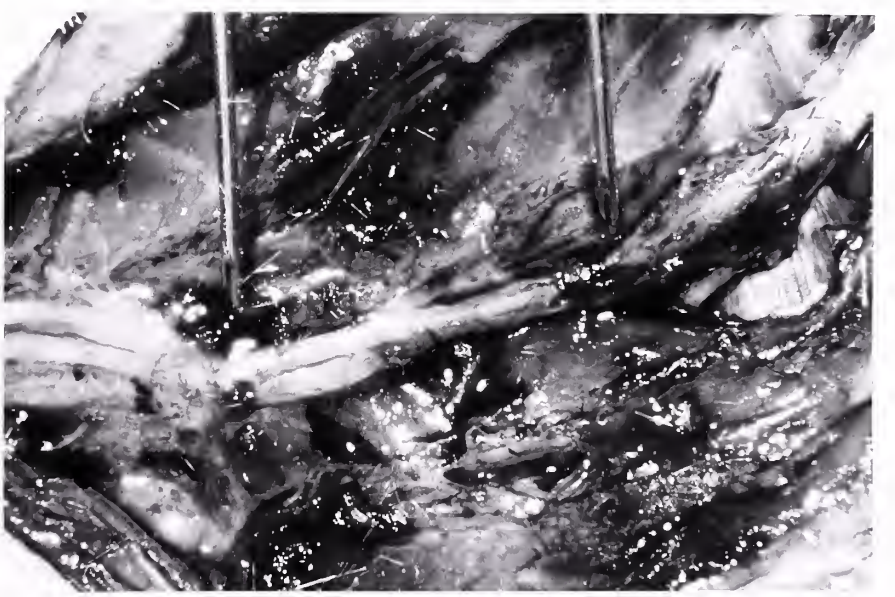


FIGURE 12. Cross sectional photomicrograph of a cryopreserved grafted sciatic nerve taken three months after grafting in the same region as the control pictured in Figure 10. A population of well myelinated nerve fibers is noted as in Figure 11.

FIGURE 13. Initial re-exploration three months after implantation of a fresh nerve autograft. Note the minimal fibrotic proliferation which allowed easy harvest of the nerve graft segment.

Figure 14. A 2.0 cm sciatic nerve cropreserved autograft (marked by pins) three months aftter inplantation. Again note the minimal fibrosis.



FIGURES 15,16 An example of a gap control rat three months after the initial procedure. Note the marked atrophy in the operative left leg. This animal had a measured flexion contracture of 40 degrees. Atrophy and flexion contractures of this magnitude were not seen in the fresh or cryopreserved grafted animals.

FIGURES 17,18. Tibialis anterior (above) and extensor digitorum longus (below) muscles in the operative (Figure 16) and nonoperative (Figure 17) legs of a rat from the gap control group. Note the marked decrease in muscle mass in the operative leg.

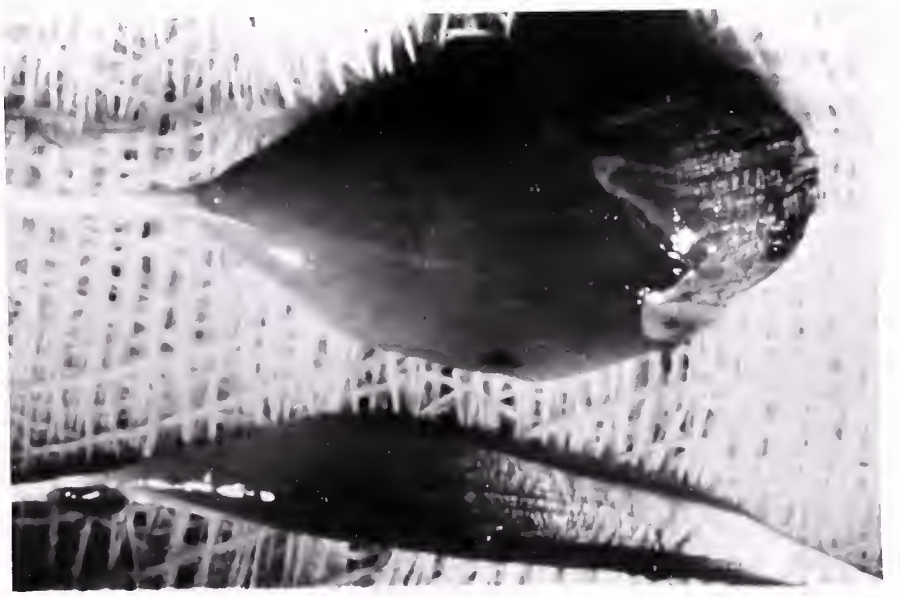


FIGURE 19. Self mutilation seen most frequently in the operative feet of gap control animals. This animal has lost its lateral three toes.

FIGURE. 20.

Fig. 20. Linear regression model of the relationship between Hydroxyproline concentration and wet Tibialis Anterior muscle weight at the time of sacrifice.

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